REMARKS

Status of the Claims

Claims 25-29, 43, and 45-47 are pending and claims 43 and 45-47 are under consideration in this application, claims 25-29 having been withdrawn for allegedly being drawn to a separate invention. All the claims under consideration stand rejected.

Claims 45 and 46 are amended herein. The amendments to claims 45 and 46 are supported by the instant specification (*e.g.*, at page 19, second paragraph) and claim 21 as originally filed and add no new matter.

No claims are added or cancelled herein and so, after entry of the present response, claims 25-29, 43, and 45-47 will be pending and claims 43 and 45-47 will be under consideration in this application.

Rejections under 35 U.S.C. § 103(a)

(a) Claims 43 and 45 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Varon *et al.*, *J. Neurotrauma* 11:473-486, 1994 in view of Kamata *et al.*, *Microbiol. Immunol.* 38:421-428, 1994. Applicants respectfully disagree with the rejection.

According to the Office Action, Varon *et al.* "teaches that neurotrophic factors are well recognized for their important function on developing neurons of the PNS, and that said factors were previously proposed to play additional and equally important roles in the CNS" (at page 4). The Office Action also cites Varon *et al.* for disclosing "that much less attention has been given to the involvement of neurotrophic factors in the CNS regenerative processes" (at page 4). The Office Action further cites Varon *et al.* for teaching "various model systems utilizing *in vivo* administration of NGF to promote neuronal outgrowth in the CNS in a spinal cord injury model" and that continuous infusion of NGF at a site of surgery "resulted in dramatically enhanced sensory fiber elongation into the injury site" (at page 4). The Office Action then concludes that "[u]pon reading the disclosure of the Varon *et al.* reference, the skilled artisan would have

recognized the desirability of developing improved methods of treating traumatic spinal cord injury" (at page 4).

The Office Action cites Kamata *et al.* as teaching "chick dorsal root ganglia (DRG) induced nerve outgrowth via administration of C botulinum C3 exoenzyme ADP-ribosyltransferase (CART) that is at least as effective as DRG outgrowth induced via the neurotrophic factor NGF" (at page 5).

The Office Action also opines that based on the teachings of Varon *et al.* and Kamata *et al.*, "the skilled artisan would have known that CART is a neurotrophic factor that is at least as effective as NGF and that neurotrophic factors are useful in providing neurite outgrowth in spinal cord injury" (at page 5). The Office Action concludes that "it would have been obvious to the person of ordinary skill to try methods of increasing neurite regeneration in the CNS via administration of CART to a patient after spinal cord injury in an attempt to provide an improved method of treating SCI" (at page 5). Applicants respectfully disagree.

Applicants have amended claims 45 and 46 to recite, in part, "[a] method to increase CNS neurite regeneration" comprising "delivering to a CNS nerve growth environment" (emphasis added). At the outset, Applicants point out that there is no evidence in Kamata *et al.* that a C3 ADP-ribossyl transferase ("CART") would promote neurite outgrowth in a CNS environment, as is required by the instant amended claims. The dorsal root ganglia (DRG) treated in Kamata *et al.* (*see, e.g.*, page 422, column 2, second paragraph; and page 424, second paragraph) are in the peripheral nervous system (PNS) and not the central nervous system (CNS) (*see, e.g.*, Varon *et al.*, page 483, paragraph 2).

It is well established that the microenvironment and cellular makeup of the CNS are very different from those of the PNS, and that factors within the CNS microenvironment, including CNS myelin, act to inhibit nerve regeneration (*see* Aguayo *et al.*, *J. Exp. Biol.* 95:231-240, 1981;

Carbonetto *et al., J. Neurosci.* 7:610-620, 1987; copies of which are enclosed as Exhibits A and B, respectively; see also page 1, first paragraph of the instant specification). Indeed, as discussed in the instant specification, "[a]n important barrier to regeneration is the axon growth inhibitory activity that is present in CNS myelin and that is also associated with the plasma membrane of oligodendrocytes, the cells that synthesize myelin in the CNS" (instant specification at page 1). Accordingly, one of ordinary skill in the art, reading Kamata *et al.*'s disclosure of a PNS model, would have no expectation of success in using a CART to increase neurite regeneration in the CNS, as recited in the pending claims.

Further, the entire disclosure of Kamata *et al.* is directed to *in vitro* assays. Specifically, Kamata *et al.* investigated the effect of CART on various *cultured* cell lines *in vitro* (*see* page 424, Table 1), and on a *cultured* dorsal root ganglion *in vitro* (*i.e.*, a PNS *in vitro* system; *see* page 424, paragraph 2, and Fig. 2). Given that not only are results observed in a PNS system not necessarily indicative of results in the CNS, but that *in vitro* results are not necessarily indicative of *in vivo* effects (especially in the CNS), one of ordinary skill in the art, reading Kamata *et al.*, would not arrive at the pending claims. Furthermore, even if Kamata *et al.* did lead one of ordinary skill in the art to the *in vivo* use of a CART (which it does not), there certainly would be no guidance to the use of a CART within the CNS *in vivo*; at most, Kamata *et al.* would lead to PNS applications.

Additionally, the Office Action's characterization of Kamata *et al.* as "concluding from their studies that [CART] exoenzyme is a neurotrophic agent" is overbroad. In fact, what Kamata *et al.* teaches is:

1. "The [CART] exoenzyme evoked the outgrowth of neurites from chick ganglion as effectively as nerve growth factor, *suggesting* that [CART] exoenzyme *possesses neurotropic activity*" (abstract, emphasis added).
2. "To confirm the *neurite-inducing activity* of [CART] exoenzyme, the neurite-outgrowth assay was performed with chick ganglia. As shown in Fig. 2, [CART]

exoenzyme evoked the formation of neurites at a relatively high concentration of the exoenzyme as compared with the effective concentration of nerve growth factor" (at page 424, paragraph 2, emphasis added).

3. "It *could be*, therefore, said that [CART] exoenzyme is a neurotropic agent" (at page 427, paragraph 1, emphasis added).

The crux of the disclosure of Kamata *et al.*, therefore, is that CART demonstrated neurite-inducing activity in a PNS culture system (*i.e.*, DRG cultures) and not, contrary to the Office Action, that CART is a neurotrophic factor.

As described by Holtzman *et al.* (*West. J. Med.* 161:246-254, 1994, a copy of which is enclosed as Exhibit C), "[n]eurotrophic factors are polypeptides that exert their actions through binding and activating specific cell surface receptors. It is increasingly apparent that neurotrophic factors have an important role in the growth, development, and maintenance of neurons in both the central and peripheral nervous systems." Holtzman *et al.* further states that neurotrophic factors belong to several defined classes (*see* page 246, Table 1). Neurotrophic factors are known to act by binding to particular cell surface receptors. For example, the members of the neurotrophin class, which includes NGF, bind to specific trk receptors (*see* page 247, Figure 1).

Nevertheless, the Office Action focuses on the term "neurotrophic factor", as used in Kamata *et al.*, and regards Kamata *et al.* as concluding that CART is a neurotrophic factor. Kamata *et al.* certainly does not state or suggest that CART belongs to a family of neurotrophic factors. Rather, as discussed above, what Kamata *et al.* discloses is that CART can induce neurite outgrowth in a specific PNS culture system.

Further, the Office Action appears to view all neurotrophic factors as interchangeable such that CART could be exchanged with any of a number of neurotrophic factors, such as NGF.

However, this is not the case. A CART is an exogenous enzyme derived from bacteria that does not bind to specific receptors. Rather, CARTs specifically ADP-ribosylate and inhibit the action of the rho family of GTP-binding proteins (*see, e.g.*, the instant specification at pages 11-12). In contrast, NGF is an endogenous polypeptide that is produced by the target of innervating neurons and that acts on receptors located on the cell surface of neurons (*see* Holtzman *et al.*, page 247). Given that the members of the family of neurotrophic factors are structurally and functionally distinct from a CART, one of ordinary skill in the art would not consider CARTs and neurotrophic factors, such as NGF, to be interchangeable.

Moreover, the Office Action cites Kamata *et al.* as teaching that DRG nerve outgrowth induced by CART "is at least as effective as DRG outgrowth induced via the neurotrophic factor NGF" (at page 5). The Abstract of Kamata *et al.* states that CART "evoked the outgrowth of neurites from chick ganglion as effectively as nerve growth factor". However, as clearly shown and focused on by Kamata *et al.*, CART was far less efficient than NGF at evoking the outgrowth Thus, Kamata *et al.* demonstrates that CART "evoked the formation of neurites at a relatively high concentration of the exoenzyme as compared with the effective concentration of nerve growth factor" (at page 424, column 1,last sentence of paragraph 2, emphasis added). Indeed, Kamata *et al.* reports that it took a 50-fold increase in the amount of CART relative to NGF to induce neurite outgrowth of cultured chick embryonic ganglia (*see* page 424, Fig. 2). Kamata *et al.* concluded that the high dose of CART was necessary for induction of neurite outgrowth due to the extremely poor ability of cells to internalize CART (*see* page 427, paragraph 2). Given that an *in vitro* PNS system required a significantly higher concentration of CART compared to NGF to induce neurite outgrowth, one of ordinary skill in the art certainly would not have considered that CART and NGF would be interchangeable in an *in vitro* PNS system, much less interchangeable for administration to the CNS of a patient.

Finally, Kamata *et al.* teaches away from the claimed methods. Kamata *et al.* states that CART induces "dysfunctional" changes in the cytoskeleton of cultured cells (at page 427,

column 2, paragraph 2). This teaching that CART resulted in abnormal cellular changes would most certainly dissuade one of ordinary skill in the art from using a CART in a patient, as currently claimed.

In light of the arguments presented above, Applicants respectfully submit that one of ordinary skill in the art would not, in view of the cited references and his or her own common sense, have had a good reason to pursue a CART as an agent to replace NGF in the method described by Varon *et al.* In addition, in view of the dearth of experimental evidence in support of the use of a CART in treating CNS injuries, such an approach would have had no reasonable expectation of success.

Therefore, the cited references, either alone or in combination, cannot render claims 43 or 45 obvious.

Finally, Applicants remind the Examiner of the unexpected results described in Example II of the instant application and referred to on page 9, second last paragraph, of the Response submitted May 24, 2007.

(b) Claims 46 and 47 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Varon *et al.* in view of Kamata *et al.*, and further in view of U.S. Patent No. 5,134,121 to Mobley *et al.* ("the '121 Patent"). Applicants respectfully traverse the rejection.

The teachings of Varon *et al.* and Kamata *et al.* have been discussed supra. The '121 patent describes NGF factor peptides that can act as NGF agonists and antagonists. None of the three cited references discloses or even suggests CART fragments, let alone Rho-family inhibitory fragments of CART, for any use, and certainly not for treating CNS injuries as in claims 46 and 47. Therefore, given that the cited references do not disclose each and every element of the claims at issue, they cannot render claims 46 and 47 obvious.

Moreover, even if the cited references did disclose each and every element of the claims (which they clearly do not), one of ordinary skill in the art, reading the cited references, would not use Rho-family inhibitory CART peptides to treat CNS injuries. As pointed out above, none of the references discloses CART fragments at all. The Office Action opines that, because the '121 patent teaches peptide fragments of NGF in the treatment of neurological diseases, it would have been obvious in view of all three references to use fragments of CART for this purpose. Applicants respectfully disagree with this position.

First, as pointed out above, one of ordinary skill in the art would not have considered using a full-length CART for the treatment of CNS injuries. Applicants respectfully submit that one of ordinary skill in the art would have been even less likely to have considered using CART fragments, particularly Rho family inhibitory fragments, for this purpose.

In addition, while the '121 patent does disclose NGF peptides that could be useful in treatment of neurological diseases, it provides no hint of using a CART, let alone CART fragments (such as Rho family-inhibitory fragments) for this purpose. Given the structural and functional differences between NGF and CARTs discussed above, a teaching that NGF fragments could be used to treat neurological diseases says nothing about the use of CART fragments. Success with NGF fragments, without more, would not have led one of ordinary skill in the art to use fragments of any other molecule, let alone a CART, in the claimed methods.

Therefore, the cited references, either alone or in combination, cannot render claims 46 or 47 obvious.

In view of the above considerations, Applicants respectfully request withdrawal of the rejections under 35 U.S.C. § 103(a).

CONCLUSION

In summary, for the reasons set forth above, Applicants maintain that the claims under consideration patentably define the invention. Applicants request that the Examiner reconsider the rejections as set forth in the Office Action, and permit the claims under consideration to pass to allowance.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicants' undersigned representative can be reached at the telephone number listed below.

Enclosed is a request for an automatic extension of time. Please charge the fee for the extension of time and any other fees or make any credits to Deposit Account No. 06-1050, referencing Attorney Docket No. 12552-002001.

Respectfully submitted,

Date: 2/21/08

/Stuart Macphail/

Stuart Macphail, Ph.D., J.D.
Reg. No. 44,217

Fish & Richardson P.C.
Citigroup Center
52nd Floor
153 East 53rd Street
New York, New York 10022-4611
Telephone: (212) 765-5070
Facsimile: (212) 258-2291

EXHIBIT A

J. exp. Biol. (1981), **95**, 231–240
With 5 figures
Printed in Great Britain

INFLUENCES OF THE GLIAL ENVIRONMENT ON THE ELONGATION OF AXONS AFTER INJURY:

TRANSPLANTATION STUDIES IN ADULT RODENTS

BY ALBERT J. AGUAYO, SAMUEL DAVID AND GARTH M. BRAY,
Neurosciences Unit, The Montreal General Hospital and McGill University

SUMMARY

Tissue transplantation methods, previously used to study neural development, myelination and inherited disorders of myelin can be applied also to the investigation of repair and regeneration in the mammalian CNS. The elongation of axons from injured peripheral nerve or CNS has been studied in adult mice and rats by observing the growth of axons into PNS or CNS tissue grafts.

Following spinal cord injury and also after transplantation of optic nerves into the PNS there is axonal sprouting but these neuronal processes fail to elongate more than a few mm into the surrounding glia. On the other hand if segments of a peripheral nerve are grafted into the transected spinal cord, axons arising from spinal neurons and dorsal root ganglia become associated with the transplanted Schwann cells and elongate along the graft, approximately 1 cm. Recently the elongation of axons from spinal and medullary neurones was studied using a new experimental model which employed PNS grafts as 'bridges' to connect the spinal cord and the brain stem. In a series of adult C57BL/6J mice and Sprague Dawley rats, autologous segments of sciatic nerve were used to create 'bridges' between the lower cervical or upper thoracic spinal cord and the medulla oblangata. The spinal cord between these two levels was left intact.

Grafted segments examined by light and electron microscope 1–7 months after surgery were well innervated by Schwann cell ensheathed axons that had grown the entire length of the graft (2 cm in mice and 3·5 cm in rats). The origin and termination of these axons were determined by transecting the regenerated grafts and applying horseradish peroxidase to the cut ends. Retrogradely labelled neurones were found to be distributed widely in the gray matter of the spinal cord and medulla near the sites of insertion of the graft. Anterogradely labelled fibres coursing within the graft penetrated the CNS for short distances, approximately 2 mm.

These new results indicate that following CNS injury a conducive glial environment does allow spinal and brain stem neurones to elongate axons for distances that can be greater than those they usually extend for in the intact animal. This evidence that the regenerative response of similar axons differs in CNS and PNS neuroglia supports the hypothesis that influences arising from the environment play an important role in the success or failure of regeneration. The regenerative potentiality of central neurones may be expressed only when the CNS neuroglial environment is changed to resemble that in the PNS.

INTRODUCTION

Functional recovery from injury to the nervous system involves the restitution of damaged structures or their substitution by alternate neuronal pathways (Eidelberg & Stein, 1974; Schneider, 1979). Such reorganizations of neuronal activity may involve the regrowth of interrupted fibres and the extension of collateral branches from intact nerve cells (Liu & Chambers, 1958; Goldberger & Murray, 1978). To become effective, these responses often require substantial elongation of neuronal processes to establish appropriate terminal connections. Axons transected in the peripheral nervous system successfully re-grow over long distances. On the other hand, although initial regenerative responses are well documented for the injured central nervous systems of most animals (Björklund & Stenevi, 1979; Cotman & Nadler, 1978; Crutcher, Brothers & Davis, 1981; Raisman & Field, 1973), the subsequent axonal growth is generally limited to a few mm and regeneration is aborted (Cajal, 1928). Although other mechanisms may contribute to the failure of regeneration in the brain and spinal cord (Guth, 1974; Varon, 1977; Lund, 1978; Grafstein & McQuarrie, 1978; Veraa & Grafstein, 1981), the limited elongation of axons within the damaged CNS appears to be a fundamental element. In this review, we summarize experimental evidence suggesting that differences in the capacity of regenerating axons to elongate in the PNS and CNS may be more dependent on the environment in which these axons are located than upon the intrinsic properties of their neurones, a role for the neural environment that was postulated by Cajal (1928).

The neural environment and axonal growth

The hypothesis that the neural environment (Table 1) plays an important role in axonal regeneration implies that axons fail to elongate in the CNS because this environment either lacks the growth-promoting properties of the PNS or exerts inhibitory influences. The different cellular responses to nerve fibre interruption in the CNS and PNS support this concept. In peripheral nerves, axons regenerate along columns of Schwann cells surrounded by basal lamina, the so-called bands of Büngner. In the adult mammalian CNS, on the other hand, no such well-aligned structures as the bands of Büngner persist after injury; rather, intertwining cytoplasmic processes of glial cells form a dense mesh or scar at the site of injury.

Conditions in the neural environment may also contribute to the more successful regrowth of axons observed in amphibians, fish and newborn mammals. In ***amphibians and fish***, CNS regeneration is usually more effective than in mammals (Murray, 1976; Michel & Reier, 1979; Forehand & Farel, 1979; Wood & Cohen, 1979). In these submammalian species, the glial cells may guide and facilitate axon growth and regeneration (Singer, Nordlander & Egar, 1979; Katz & Lasek, 1979; Wood & Cohen, 1979). In ***newborn mammals***, the transection of CNS fibre tracts can be followed by a growth of axons over distances greater than those observed after injury in older animals (Kalil & Reh, 1979), although some of the axons observed in these experiments may have been due to the arrival of developing fibres rather than regeneration. The enhanced elongation in these animals may reflect the paucity of glial cells in young animals (Brizzee & Jacobs, 1959; Fulcrand & Privat, 1977) and/or developmental changes in the properties of these cells, including their tendency to form

Table 1. *Components of the neural environment*

	PNS	CNS
Sheath cells	Schwann cells	Oligodendrocytes, astrocytes
Other cells	Fibroblasts, mast cells	Microglia
Extracellular matrix	Basal lamina, collagen	—
Diffusable factors*	?	?

* Although diffusable factors that influence neurite growth have been recognized *in vitro*, the role of such substances in regeneration has not been established *in vivo*.

less dense glial scars (Sumi & Hager, 1968). Furthermore, regeneration in newborn animals may also be more successful because of the relatively short distances that are required for axons to reach their targets.

The growth facilitating effects of various components of the neural microenvironment have been investigated in tissue culture. Surface characteristics of the substrate such as its adhesiveness, can influence branch formation and the course of axonal growth in vitro (Letourneau, 1975; Sidman & Wessells, 1975). Furthermore, Schwann cells may have trophic effects on neurones (Varon & Bunge, 1978). In dissociated cultures, Schwann cells and fibroblasts can substitute for nerve growth factor (NGF) to support the survival and neuritic growth of sympathetic or dorsal root ganglion cells (Burnham, Raiborn & Varon, 1972). *In vitro* studies have also demonstrated factors other than NGF have trophic influences on specific cell populations (Edgar, Barde & Thoenen, 1981). Such factors can be derived from glial tumour cells (Monard *et al.* 1975), Schwann cells (Varon, Skaper & Manthorpe, 1981) or cardiac muscle cells (Adler & Varon, 1979; Edgar *et al.* 1981). The requirements of cultured neurones for some of these factors changes during development (Barde, Edgar & Thoenen, 1980).

The use of experimental transplants to study regeneration and neural repair

Tissue transplantation techniques used to study neural development (Harrison 1934; Harris, 1979), myelination (Aguayo, Charron & Bray, 1976) and inherited disorders of myelin formation (Aguayo, Bray & Perkins, 1979), have also been applied to the investigation of repair and regeneration in the mammalian nervous system. Two main strategies are available:

(*a*) *Neural tissue transplants* in which neurones from foetal or newborn animals survive and may establish connections with the brains of adult hosts (Stenevi & Björklund, 1978; Kromer, Björklund & Stenevi, 1979; Perlow *et al.* 1979; Rosenstein & Brightman, 1979);

(*b*) *Grafts of central or peripheral glia* into the peripheral nerves or central nervous system of adult animals (Aguayo *et al.* 1979). Such grafts were employed to assess interactions of axons from host neurones in the PNS or CNS with transplanted glia or Schwann cells to compare the effects of different environments on axonal growth (Aguayo *et al.* 1979).

In the present report we review the results of experiments in which we have used this approach to assess influences that favour or impair axon elongation after injury.

Transplantation of Schwann cells into peripheral nerves. Allogenic and xenogenic nerve segments or *in vitro* preparations of Schwann cells were transplanted into the transected sciatic nerves of immune-suppressed mice where they ensheathed and

myelinated axons regenerating from the host nerve (Aguayo *et al.* 1977*a*, *b*). When after regeneration of the grafted nerves, the immune suppression was discontinued the allo- or xenogenic sheath cells were rejected and the segments of nerve at the site of the original graft became ensheathed by Schwann cells migrating from the host (Aguayo *et al.* 1977*b*, 1979). This Schwann cell migration may have been guided and facilitated by the axons and basal lamina that survived the rejection of the grafted (donor) Schwann cells. Thus, the transplanted Schwann cells had acted as temporary 'bridges' to facilitate axonal regrowth across the nerve gaps. The recent finding that Schwann cells grown *in vitro* can be transplanted into peripheral nerve, as well as spinal cord (Duncan *et al.* 1981), provides a new experimental technique for the study of cell interactions in the PNS and CNS and also suggests that it may be possible to use cells cultured *in vitro* for sheath cell replacement and experimental neural repair.

Transplantation of peripheral nerve segments into the CNS. Although Cajal (1928) and several other investigators (Tello, 1911; Sugar & Gerard, 1940; Clark, 1943; Kao, Chang & Bloodworth, 1977) demonstrated the reinnervation of peripheral nerve segments grafted into the spinal cord, it remained to be determined whether any axons within these grafts were derived from intrinsic spinal neurones or if all were the result of regrowth from neighbouring spinal roots (Sugar & Gerard, 1940; Clark, 1943). The experiments described below were designed to answer this question using contemporary morphologic techniques.

In adult female rats, a segment of the thoracic spinal cord was resected and replaced by an autologous sciatic nerve graft 1 cm long. Ten days to four months later, the animals were killed and the graft and its junctions with the spinal cord were examined by light and electron microscope. All grafts of more than 3 weeks duration were richly innervated with myelinated and unmyelinated axons, even if the dorsal spinal roots entering the graft site and their ganglia had been avulsed. At the junction between the graft and the central nervous tissues, myelinated and unmyelinated axons were observed within dome-shaped structures containing astrocytic processes and surrounded by basal lamina. Occasional nodes of Ranvier were seen in which one heminode had peripheral myelin and the other central myelin.

Retrograde cell labelling with horseradish peroxidase (HRP) (Mesulam, 1978) indicated that some of the axons within the graft originated from intrinsic spinal cord neurones and others were derived from the dorsal root ganglia below the graft (Richardson, McGuinness & Aguayo, 1980). In another group of animals, a tritiated leucine-proline mixture was injected into the lumbar dorsal root ganglia three months after grafting a peripheral nerve segment into the transected thoracic spinal cord. Radioautography showed that anterogradely labelled axons originating in the dorsal root ganglia several segments below the level of the graft had ascended in the dorsal columns and entered the graft. Some of these labelled axons crossed the grafts from the caudal end of the cord but did not appear to re-enter the rostral spinal stump (Richardson, Aguayo & McGuinness, 1981). These experiments indicate that axons from dorsal root ganglia as well as from spinal neurones in the proximity of the spinal cord transection are capable of growth along the PNS grafts for distances of approximately 1 cm. These axons may have originated by regrowth of damaged fibres or from the extension of collaterals from uninjured neurones.

Transplantation of CNS glia into peripheral nerves. Interactions between regenerating

peripheral axons and transplanted CNS glia were studied in mice and rats by grafting segments of optic nerve 5 mm in length into the main trunk of the sciatic nerve or one of its branches (Aguayo *et al.* 1978; Weinberg & Spencer, 1979). Such optic nerve grafts contain astrocytes and oligodendrocytes but not neurones. Following transplantation the axons within the optic nerve segments degenerated but (in contrast to the rapidity with which myelin and axon remnants were cleared from degenerating peripheral nerves) such debris was observed in the grafts for several months. The majority of axons arising from the proximal stump of the recipient peripheral nerve bypassed the transplant and re-entered the distal stump. However, the glial transplants were penetrated by some peripheral axons that became ensheathed by astrocytic processes and were occasionally myelinated by oligodendrocytes. The longitudinal growth of most of these axons was limited to less than 1 mm with only a few axons reaching the distal end of the graft.

The results of these experiments suggest four conclusions: (i) CNS transplants are less receptive to the regeneration of peripheral axons than are peripheral nerve grafts or the distal stumps of transected peripheral nerves; (ii) the connective tissue that forms at the margins of the grafts is not an impenetrable barrier to axon growth because some axons enter the glial grafts although they fail to continue to elongate within them; (iii) glial cells survive in the graft and are able to ensheath and myelinate penetrating axons; (iv) early synaptogenesis with neurones, a phenomenon shown in experiments involving transected spinal cords (Bernstein & Bernstein, 1971), could not be the cause of the limited axonal growth observed in these grafts because the transplanted optic nerve segments contained no nerve cells.

The observations in the optic nerve grafts are also in concordance with the demonstration that injured dorsal root axons regenerate as far as the PNS–CNS boundary but only a few enter the spinal cord (Stensaas, Burgess & Horch, 1979; Perkins *et al.* 1980).

Peripheral nerve grafts as 'bridges' for regenerating CNS axons

The sciatic and optic nerve graft experiments described above provided the basis for more recent studies designed to determine the potential of axons injured in the CNS to elongate for distances equivalent to some of the long projecting neurone systems in the intact CNS (David & Aguayo, 1981).

Autologous segments of the sciatic nerve (2 cm in mice and 3·5 cm in rats) were used to prepare 'bridges' between the medulla oblongata and the lower cervical or upper thoracic spinal cord in a series of adult C57BL/6J mice and Sprague–Dawley rats. The bridging nerve grafts were placed extraspinally within the subcutaneous tissues in the back of the animals (Fig. 1). Through a small laminectomy an incision was made with a 150 μm glass rod into the dorsal spinal cord and medulla to insert the ends of the nerve 'bridges'. The spinal cord between these two levels was left intact. Animals survived without apparent neurologic deficits and were sacrificed between 1 and 7½ months after grafting.

Examination of the grafts by light and electron microscope showed that they were well innervated by axons ensheathed by Schwann cells (Fig. 2). The origin and termination of these axons was determined by transecting the regenerated grafts and applying horseradish peroxidase to the cut ends (Mesulam, 1978; Richardson *et al.*

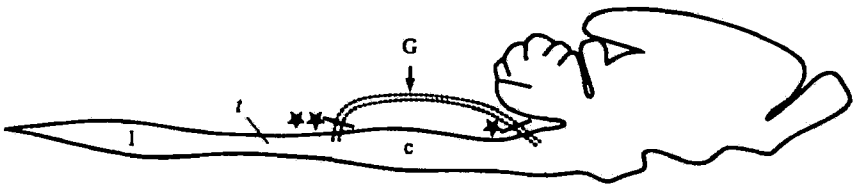

Fig. 1. Diagram of sagittal section of rat CNS with a peripheral nerve graft (G) linking the medulla oblongata and the upper thoracic spinal cord (t). The elongation of axons was measured as the distance between the site of HRP application and the labelled cells in the CNS. For this purpose, when neurones were sought in the medulla the tracer was applied to the nerve 30 mm from the brain stem (**). Conversely, when the growth of spinal neurones was investigated, the graft was cut close to the brain stem (*). Anterograde labelling was obtained by applying HRP to the short stumps.

1980). Retrogradely-labelled neurones were found in several nuclei of the medulla and in the gray matter of the spinal cord in areas that were close to the insertion of the graft (Figs. 3, 4). Anterogradely-labelled fibres penetrated the CNS for distances that did not exceed 2 mm (Fig. 5). The results of these experiments indicate that the PNS 'bridges' contain axons from spinal cord and brain stem neurones. Under such experimental conditions, such axons are capable of a growth that approximates 1·5 cm in mice and 3 cm in rats, distances that are equal to or greater than the lengths of axons from many of these neurones in intact animals.

This new experimental approach has several advantages that are important for the study of the origin, course, length and termination of regenerating axons:

(i) because most of each peripheral nerve 'bridge' is located extraspinally, there is little risk that the HRP applied to the transected nerve to identify cells in the spinal cord and brain stem could reach the CNS by interstitial spread and label neurones spuriously;

(ii) by selectively positioning the nerve 'bridges', it is possible to direct the course of axons from and into specific regions of the CNS including the cerebral hemispheres (Benfey & Aguayo, 1981);

(iii) the length of axonal growth can be determined by measuring the distance between the level of HRP application and the labelled cell bodies;

(iv) the terminations of regenerated axons can be visualized by anterograde labelling;

(v) because experimental animals are not paralysed and retain bowel and bladder function after this procedure, in contrast to the animals grafted after a complete spinal cord transection, their care and survival is greatly facilitated.

DISCUSSION

The failure of axons to regenerate as effectively in the mammalian CNS as in peripheral nerves has been attributed to several mechanisms including a primary inability of CNS neurones to support the regrowth of their processes, early synapse formation with other neurones (Bernstein & Bernstein, 1971), a blocking effect of the astrocytic and connective tissue scar (Windle, 1956), autoimmunity (Berry & Riches, 1974) and differences between the blood-brain and blood-nerve barriers (Kiernan, 1979). Without excluding the possibility that these mechanisms are operative, our approach has been to investigate the general conditions that influence the axons of CNS neurones

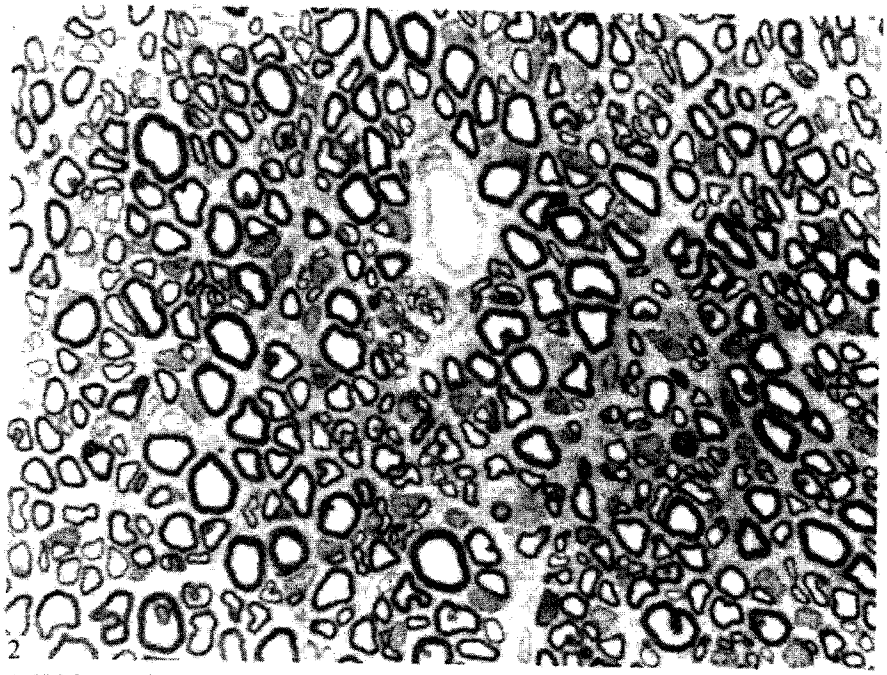

Fig. 2. Cross-section of a peripheral nerve graft at the mid point between the brain stem and spinal cord. The axons are normally ensheathed by Schwann cells and myelin (light micrograph, × 1060).

Fig. 3. Two labelled neurones in the nucleus reticularis lateralis are seen in the medulla after HRP was applied to the caudal end of the graft, 30 mm from the brain stem (dark field micrograph, × 530).

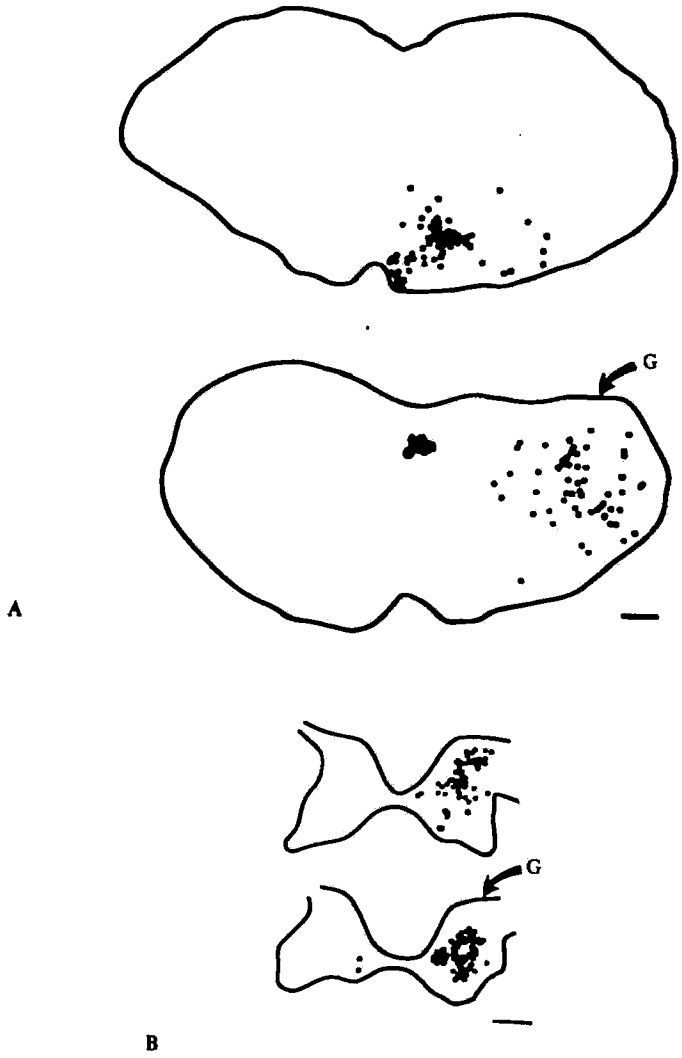

Fig. 4. Diagrams illustrating the position of HRP labelled neurones in (A) medulla and (B) spinal cord after HRP application to the graft.

elongate *in vivo*. Our experiments have demonstrated that while PNS axons with a known capacity to regenerate fail to elongate in a CNS glial environment (Aguayo *et al.* 1978), the axons from intrinsic CNS neurones will grow into segments of peripheral nerves (Richardson *et al.* 1980; David & Aguayo, 1981; Benfey & Aguayo, 1981). The studies of peripheral nerve 'bridges' (David & Aguayo, 1981) also indicate that axons in the injured CNS are able to elongate for unprecedented distances within such peripheral nerve grafts. Although regenerating axons only penetrate damaged CNS tissues for short distances, it would appear that glial cells within the denervated CNS remain responsive to the influences of regenerating axons and are capable of producing myelin (Aguayo *et al.* 1978; Weinberg & Spencer, 1979).

Several questions raised by these studies of CNS regeneration in the adult mammalian CNS require further exploration:

(i) Do the axons that elongate within the PNS grafts originate by regrowth of damaged CNS fibres, by sprouting from uninjured nerve cells or by both mechanisms?

(ii) Are different neuronal populations capable of the same regenerative responses? It is not known if the neurones that have been identified in these experiments represent a special group of cells or if they are examples of a general rule that applies to all neurones.

(iii) Are long pathways, such as the cortiocospinal tract, capable of axonal regrowth?

(iv) Are regenerating axons able to establish functional synaptic connections with neurones in the limited areas of the CNS they penetrate.

(v) Are specific chemical substances and/or the surface properties of axons and glia responsible for the differences in axonal regeneration in the peripheral and central neural environment?

If the conclusion that interactions between neurones and their glial environment play a determinant role in the failure of CNS regeneration is corroborated, the study of the molecular basis of these interdependencies may lead to experimental approaches aimed at the modification of neuronal and glial responses to CNS injury. If axons from CNS neurones are able to establish functional connections with cells in the target regions to which they have been directed, it may also be possible to devise experimental strategies that will permit selected populations of axons to bypass damaged CNS tissues and connect with specific groups of neurones at a distance.

REFERENCES

ADLER, R. & VARON, S. (1979). Cholinergic neuronotrophic factors. V. Segregation of survival and neurite-promoting activities in heart-conditioned media. *Brain Res.* **188**, 437–448.

AGUAYO, A. J., CHARRON, L. & BRAY, G. M. (1976). Potential of Schwann cells from unmyelinated nerves to produce myelin – a quantitative ultrastructural and radioautographic study. *J. Neurocytol.* **5**, 565–573.

AGUAYO, A. J., ATTIWELL, M., TRECARTEN, J., PERKINS, C. S. & BRAY, G. M. (1977*a*). Abnormal myelination in transplanted Trembler mouse Schwann cells. *Nature, Lond.* **265**, 73–75.

AGUAYO, A. J., KASARJIAN, J., SKAMENE, E., KONGSHAVN, P. & BRAY, G. M. (1977*b*). Myelination of mouse axons by Schwann cells transplanted from normal and abnormal human nerves. *Nature, Lond.* **268**, 753–755.

AGUAYO, A. J., DICKSON, R., TRECARTEN, J., ATTIWELL, M., BRAY, G. M. & RICHARDSON, P. (1978) Ensheathment and myelination of regenerating PNS fibers by transplanted optic nerve glia. *Neurosci. Lett.* **9**, 97–104.

AGUAYO, A. J., BRAY, G. M. & PERKINS, C. S. (1979). Axon–Schwann cell relationships in neuropathies of mutant mice. *Ann. N.Y. Acad. Sci.* **317**, 512–531.

AGUAYO, A. J., BRAY, G. M., PERKINS, C. S. & DUNCAN, I. D. (1979). Axon-sheath cell interactions in peripheral and central nervous system transplants. *Soc. Neurosci. Symp.* **4**, 361–383.

BARDE, Y.-A., EDGAR, D. & THOENEN, H. (1980). Sensory neurons in culture; changing requirements for survival factors during embryonic development. *Proc. natl Acad. Sci. U.S.A.* **77**, 1199–1203.

BENFEY, M. & AGUAYO, A. J. (1981). Axons from neurons in the cerebral hemispheres of adult rats grow into PNS grafts. *Can. J. Neurol. Sci.* (In the press).

BERNSTEIN, J. J. & BERNSTEIN, M. E. (1971). Axonal regeneration and formation of synapses proximal to the site of lesion following hemisection of the rat spinal cord. *Expl Neurol.* **30**, 336–351.

BERRY, M. & RICHES, A. C. (1974). An immunological approach to regeneration in the central nervous system. *Br. med. Bull.* **30**, 135–140.

BJÖRKLUND, A. & STENEVI, U. (1979). Regeneration of monoaminergic and cholinergic neurons in the mammalian central nervous system. *Physiol. Rev.* **59**, 62–100.

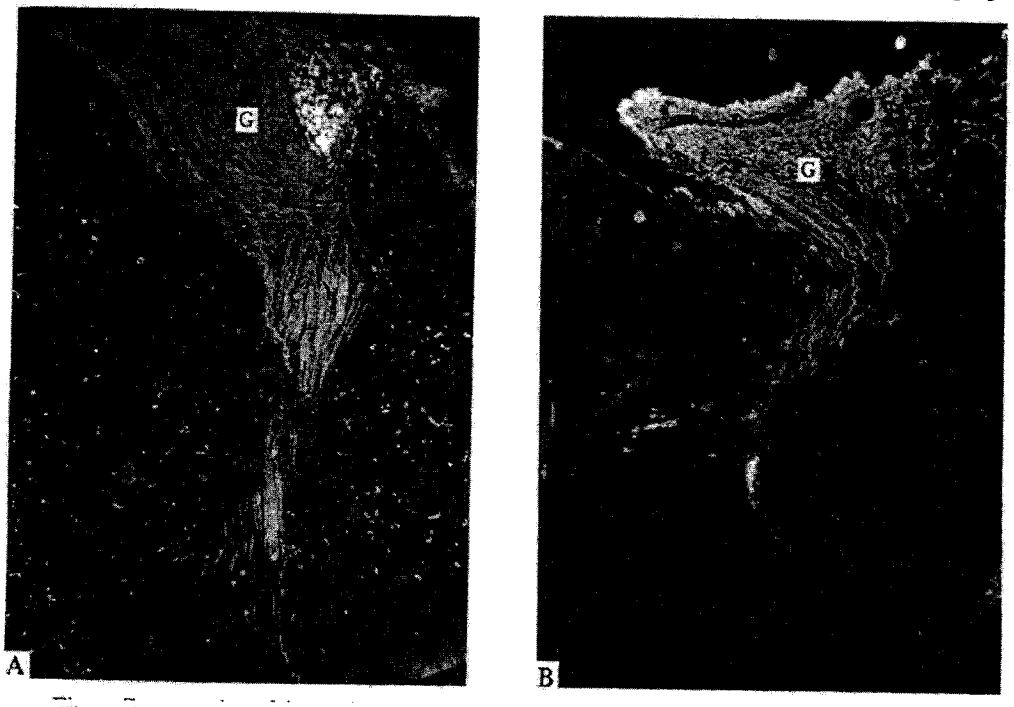

Fig. 5. Cross-section of the medulla oblongata (A) and spinal cord (B) illustrating the course of axons at the junction between the PNS graft (G) and the CNS tissues. The anterograde tracing of these axons was obtained by the application of HRP to the transected graft 5 mm away from the surface of the neuraxis.

BRIZZEE, K. R. & JACOBS, L. A. (1959). The glia neuron index in the submolecular layers of the motor cortex in the cat. *Anat. Rec.* **134**, 97–105.

BURNHAM, P. A., RAIBORN, C. & VARON, S. (1972). Replacement of Nerve Growth Factor by ganglionic non-neuronal cells for the survival *in vitro* of dissociated ganglionic neurons. *Proc. natl. Acad. Sci. U.S.A.* **69**, 3556–3560.

CAJAL, S. R. Y. (1928). *Degeneration and Regeneration of the Nervous System*, R. M. May. London: Oxford University Press.

CLARK, W. E. LEG. (1943). The problem of neuronal regeneration in the central nervous system. II. The insertion of peripheral nerve stumps into the brain. *J. Anat.* **77**, 251–259.

COTMAN, C. W. & NADLER, J. V. (1978). Reactive synaptogenesis in the hippocampus. In *Neuronal Plasticity* (ed. C. W. Cotman), pp. 227–271. New York: Raven Press.

CRUTCHER, K. A., BROTHERS, L. & DAVIS, J. N. (1981) Sympathetic noradrenergic sprouting in response to central cholinergic destruction. A histochemical study of neuronal sprouting in the rat hippocampal formation. *Brain Res.* (In the Press.)

DAVID, S. & AGUAYO, A. J. (1981). Axonal elongation into PNS 'bridges' after CNS injury in adult rats. *Science, N.Y.* (In the press.)

DUNCAN, I. D., AGUAYO, A. J., BUNGE, R. P. & WOOD, P. M. (1981). Transplantation of rat Schwann cells grown in tissue culture into the mouse spinal cord. *J. Neurol. Sci.* **49**, 241–252.

EDGAR, D., BARDE, Y-A. & THOENEN, H. (1981). Subpopulations of cultured chick sympathetic neurons differ in their requirements for survival factors. *Nature, Lond.* **289**, 294–295.

EIDELBERG, E. & STEIN, D. G. (1974). *Neurosciences Res. Prog. Bull.* **12**, no. 2.

FOREHAND, C. J. & FAREL, P. B. (1979). Restitution of long descending tracts and normal behaviour following spinal cord transection in anura. *Neurosci. Abst.* **5**, 677.

FULCRAND, J. & PRIVAT, A. (1977). Neuroglial reactions secondary to Wallerian degeneration in the optic nerve of the postnatal rat: Ultrastructural and quantitative study. *J. comp. Neurol.* **176**, 189–224.

GOLDBERGER, M. E. & MURRAY, M. (1978). Recovery of movement and axonal sprouting may obey some of the same laws. In *Neuronal Plasticity* (ed. C. W. Cotman), pp. 73–96. New York: Raven Press.

GRAFSTEIN, B. & MCQUARRIE, I. G. (1978). Role of the nerve cell body in axonal regeneration. In *Neuronal Plasticity* (ed. C. W. Cotman) pp. 155–195. New York: Raven Press.

GUTH, L. (1974). Axonal regeneration and functional plasticity in the central nervous system. *Expl Neurol.* **45**, 606–654.

HARRIS, W. A. (1979). Amphibian chimaeras and the nervous system. *Soc. Neurosci. Symp.* **4**, 228–257.

HARRISON, R. G. (1934). Heteroplastic grafting in embryology. *Harvey Lect.* **29**, 116–157.

KALIL, K. & REH, T. (1979). Regrowth of severed axons in the neonatal central nervous system: establishment of normal connections. *Science, N.Y.* **205**, 1158–1161.

KAO, C. C., CHANG, L. W. & BLOODWORTH, J. M. B. (1977). Axonal regeneration across transected mammalian spinal cords: an electron microscopic study of delayed microsurgical nerve grafting. *Expl Neurol.* **54**, 591–615.

KATZ, M. J. & LASEK, R. J. (1979). Substrate pathways which guide growing axons in Xenopus embryos. *J. comp. Neurol.* **183**, 817–831.

KIERNAN, J. A. (1979). Hypothesis concerned with axonal regeneration in the mammalian nervous system. *Biol. Rev.* **54**, 155–197.

KROMER, L. E., BJÖRKLUND, A. & STENEVI, U. (1979). Intracephalic implants: A technique for studying neuronal interactions. *Science, N.Y.* **204**, 1117–1119.

LETOURNEAU, P. C. (1975). Cell-to-substratum adhesion and guidance of axonal elongation. *Devl Biol.* **44**, 92–101.

LIU, C. N. & CHAMBERS, W. W. (1958). Intraspinal sprouting of dorsal root axons. *Archs Neurol. Psychiat.* **79**, 46–61.

LUND, R. D. (1978). *Development and Plasticity of the Brain. An Introduction.* New York: Oxford University Press.

MICHEL, M. E. & REIER, P. J. (1979). Axonal-ependymal associations during early regeneration of the transected spinal cord in *Xenopus laevis tadpoles*. *J. Neurocytol.* **8**, 529–548.

MESULAM, M.-M. (1978). Tetramethylbenzidine for horseradish peroxidase neurohistochemistry: a non-carcinogenic blue reaction-product with superior sensitivity for visualizing neural afferents and efferents. *J. Histochem. Cytochem.* **26**, 106–117.

MONARD, D., STOCKEL, K., GOODMAN, R. & THOENEN, H. (1975). Distinction between nerve growth factor and glial factor. *Nature, Lond.* **258**, 444–445.

MURRAY, M. (1976). Regeneration of retinal axons into the goldfish optic tectum. *J. comp. Neurol.* **168**, 175–196.

PERKINS, C. S., CARLSTEDT, T., MIZUNO, K. & ARGUAYO, A. J. (1980). Failure of regenerating dorsal root axons to regrow into the spinal cord. *Can. J. Neurol. Sci.* **7**, 323.

PERLOW, M. J., FREED, W. J., HOFFER, B. J., SEIGER, A., OLSON, L. & WYATT, R. J. (1979). Brain grafts

reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. *Science, N.Y.* **204**, 643–647.

RAISMAN, G. & FIELD, P. M. (1973). A quantitative investigation of the development of collateral reinnervation after partial deafferentation of the septal nuclei. *Brain Res.* **50**, 241–264.

RICHARDSON, P. M., MCGUINNESS, U. M. & AGUAYO, A. J. (1980). Axons from CNS neurons regenerate into PNS grafts. *Nature, Lond.* **284**, 264–265.

RICHARDSON, P. M., AGUAYO, A. J. & MCGUINNESS, U. M. (1981). Role of neuroglial cells in axonal regeneration. In *Proceedings First Annual Symposium on Spinal Cord Reconstruction* (ed. C. Kao, R. B. Bunge and P. Reier), New York: Raven Press. (In the Press.)

ROSENSTEIN, J. M. & BRIGHTMAN, M. W. (1979). Regeneration and myelination in autonomic ganglia transplanted to intact brain surfaces. *J. Neurocytol.* **8**, 359–379.

SCHNEIDER, G. E. (1979). Is it really better to have your brain lesion early? A revision of the 'Kennard principle'. *Neuropsychologia* **17**, 557–583.

SIDMAN, R. L. & WESSELLS, N. K. (1975). Control of direction of growth during the elongation of neurities. *Expl Neurol.* **48**, 237–251.

SINGER, M., NORDLANDER, R. H. & EGAR, M. (1979). Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt: The blue print hypothesis of neuronal pathway patterning. *J. Comp. Neurol.* **185**, 1–22.

STENEVI, U. & BJÖRKLUND, A. (1978). Transplantation techniques for the study of regeneration in the central nervous system. *Prog. Brain Res.* **48**, 101–112.

STENSAAS, L. J., BURGESS, P. R. & HORCH, K. W. (1979). Regenerating dorsal root axons are blocked by spinal cord astrocytes. *Neurosci. Abst.* **5**, 684.

SUGAR, O. & GERARD, R. W. (1940). Spinal cord regeneration in the rat. *J. Neurophysiol.* **3**, 1–19.

SUMI, S. M. & HAGER, H. (1968). Electron microscopic study of the reaction of the newborn rat brain to injury. *Acta Neuropathol.* (Barl.) **10**, 324–335.

TELLO, F. (1911). La influencia del neurotropismo en la regeneracion de los centros nerviosos. *Trab. Lab. Invest. Bioll univ. Madr.* **9**, 123–159.

VARON, S. (1977). Neural growth and regeneration: A cellular perspective. *Expl Neurol.* **54**, 1–6.

VARON, S. S. & BUNGE, R. P. (1978). Trophic mechanisms in the peripheral nervous system. *Ann. Rev. Neurosci.* **1**, 327–361.

VARON, S., SKAPER, S. D. & MANTHORPE, M. (1981). Trophic activities for dorsal root and sympathetic ganglionic neurons in media conditioned by Schwann and other peripheral cells. *Devl Brain Res.* **1**, 73–88.

VERAA, R. P. & GRAFSTEIN, B. (1981). Cellular mechanisms for recovery from nervous system injury: A conference report. *Expl Neurol.* **71**, 6–75.

WEINBERG, E. L. & SPENCER, P. S. (1979). Studies on the control of myelinogenesis 3. Signalling of oligodendrocyte myelination by regenerating peripheral axons. *Brain Res.* **162**, 273–279.

WINDLE, W. F. (1956). Regeneration of axons in the vertebrate central nervous system. *Physiol. Rev.* **36**, 426–440.

WOOD, M. R. & COHEN, M. J. (1979). Synaptic regeneration in identified neurons of the lamprey spinal cord. *Science, N.Y.* **206**, 344–347.

EXHIBIT B

The Journal of Neuroscience, February 1987, 7(2): 610–620

Nerve Fiber Growth in Culture on Tissue Substrata from Central and Peripheral Nervous Systems

Salvatore Carbonetto, David Evans, and Philippe Cochard

The Neurosciences Unit, Montreal General Hospital Research Institute, McGill University, Montreal, Quebec H3G 1A4, Canada

In adult mammals, injured neurons regenerate extensively within the PNS but poorly, if at all, within the CNS. We have studied the effect of substrata consisting of tissue sections from various nervous systems on nerve fiber growth in culture and correlated our results with the growth potential of these tissues *in vivo*. Ganglionic explants from embryonic chicks (9–12 d) fail to extend nerve fibers onto sections of adult rat optic nerve or spinal cord (CNS) but do so on sciatic nerve (PNS). Dissociated DRG neurons behave similarly whether in serum-containing or defined medium. Tissue substrata from nervous systems that support regeneration *in vivo*—i.e., goldfish optic nerve, embryonic rat spinal cord, degenerating sciatic nerve—also support fiber growth in culture. Within the same culture, neurons will grow onto sciatic nerve rather than neighboring optic nerve sections, suggesting that the responsible agent(s) is not soluble. In addition, neurons adhere more extensively to sciatic nerve substrata than to optic nerve. The occurrence of 3 molecules known to be involved in neuron–substratum adhesion and nerve fiber growth *in vitro* has been documented immunocytochemically in the tissue sections. One of these, laminin, is demonstrable in all tissues tested that supported nerve fiber growth. Immunoreactivities for fibronectin and heparan sulfate proteoglycan are found in only some of these tissues. None of these 3 molecules can be demonstrated in neural cells of normal adult rat CNS tissue. Our data suggest that these molecules may be important effectors of nerve regeneration in neural tissues.

Following trauma to the CNS of adult mammals, nerve fiber growth is meager and functional recovery correspondingly poor. In contrast, neurons of the PNS may extend nerve fibers for many centimeters, reconnecting more or less accurately with their targets. Aguayo and coworkers have shown that some axotomized CNS neurons have the capacity to regenerate their axons for considerable distances into peripheral nerve grafts (Richardson et al., 1980; David and Aguayo, 1981; Aguayo, 1985). Moreover, neither PNS (Nathaniel and Pease, 1963; Nathaniel and Nathaniel, 1973; Aguayo et al., 1979) nor CNS neurons are able to elongate into the adult CNS. Taken together, these data suggest that heterogeneity in the microenvironment of CNS and PNS neurons is largely responsible for their disparate response to axotomy.

Hypotheses regarding the molecular basis of axonal regeneration are often grounded in observations made on nerve fibers growing in culture. Cultured neurons require 2 major environmental influences to elaborate nerve fibers, growth factors (reviewed by Berg, 1984) and a properly adhesive substratum (Letourneau, 1975; Greene and Tischler, 1976). NGF, the best-known of the growth factors, is required for the survival and growth of dorsal root ganglion (DRG) and sympathetic neurons *in vivo* as well as in culture (reviewed by Greene and Shooter, 1980). Other growth factors have been identified (Collins, 1978; Barde et al., 1982), but their function *in vivo* has not been described.

Several adhesive molecules mediate the attachment of neurons to culture substrata in addition to supporting nerve fiber growth (Letourneau, 1975; Akers et al., 1981; Baron van Evercooren et al., 1982; Carbonetto et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983; Rauvala, 1984; Smalheiser et al., 1984). Three of these, the adhesive proteins laminin, fibronectin, and collagen, are components of the extracellular matrix of the nervous system and may function *in vivo* as effectors of nerve fiber growth. For fibronectin (Carbonetto et al., 1983) and laminin (Edgar et al., 1984) growing nerve fibers interact with a discrete region of the protein, as might occur during ligand receptor binding. Interestingly, laminin alone can mimic some of the trophic properties of NGF (Edgar et al., 1984; Davis et al., 1985; Lander et al., 1985), suggesting that this large multifunctional molecule has domain(s) that are neurotrophic and/or adhesive for cells. The function of these adhesive proteins during nerve regeneration *in vivo* has not been tested.

This paper describes our attempts to analyze some of the environmental influences within the nervous system affecting nerve fiber growth. In our studies, neurons were cultured on sections of neural tissues from PNS or CNS. The neurons failed to extend nerve fibers on tissue substrata from adult rat CNS (optic nerve, spinal cord), whereas extensive nerve fiber growth occurred on tissue from PNS (sciatic nerve). In addition, goldfish optic nerve and embryonic rat spinal cord, which support nerve fiber growth *in vivo*, did so in our culture system. The difference in the growth of neurons on these tissue substrata appears to reflect the inactivity or absence of adhesive molecules in the adult mammalian CNS tissues. Several extracellular matrix (ECM) components, mentioned above (laminin, fibronectin, heparan sulfate proteoglycan), that are demonstrable immunocytochemically in the PNS are essentially absent in the adult CNS.

Received Mar. 31, 1986; revised Aug. 6, 1986; accepted Aug. 11, 1986.

We thank Dr. John Hassell, NIH, for supplying antibodies to heparan sulfate proteoglycan. We also thank Dr. R. Levine for his critique of the manuscript. D.E. was a McGill University summer research fellow and P.C. the recipient of NATO and CNRS postdoctoral fellowships. This work was supported by grants to S.C. from NIH (NS19068) and MRC (MA-9000).

Correspondence should be addressed to Dr. S. Carbonetto, Montreal General Hospital Research Institute, McGill University, 1650 Cedar Avenue, Montreal, P.Q. H3G 1A4, Canada.

Copyright © 1987 Society for Neuroscience 0270-6474/87/020610-11$02.00/0

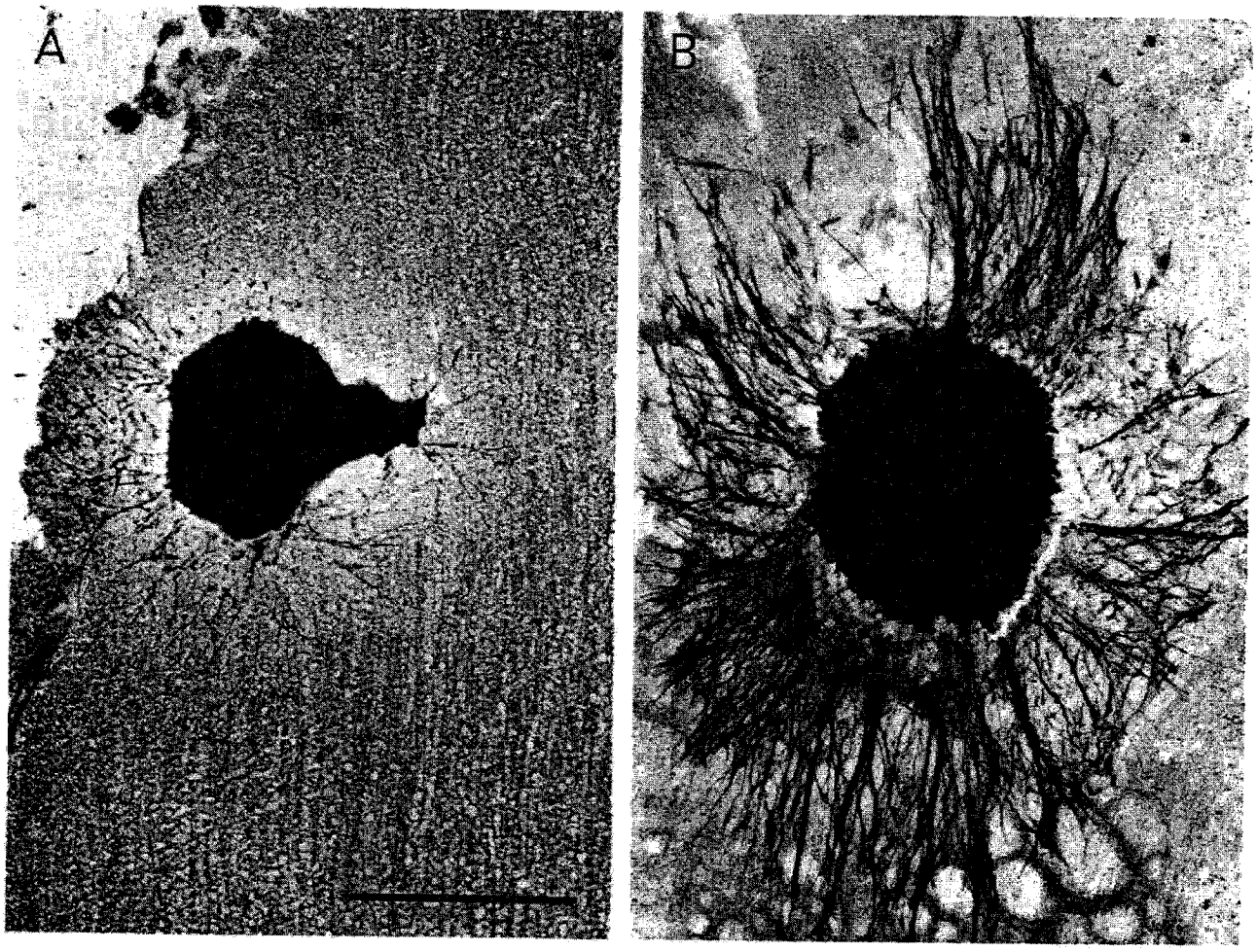

Figure 1. Growth of DRG explants on substrata from spinal cord and sciatic nerve. *A*, Ganglia attached to sections of CNS tissue generally did not grow but occasionally extended several short processes such as the "minimal" growth shown here. Calibration bar, 0.5 mm. *B*, By comparison, ganglia attached to sciatic nerve sections grew extensively (same magnification as in *A*).

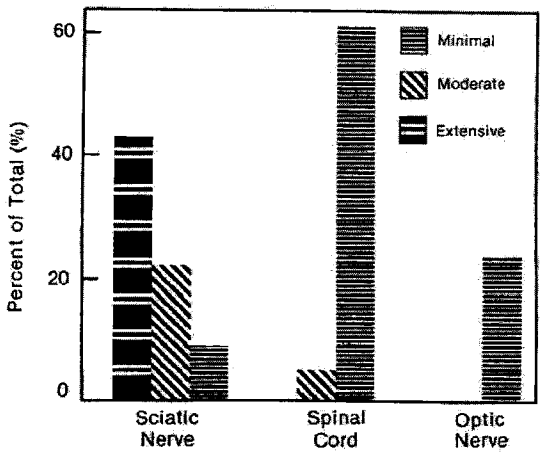

Figure 2. Nerve fiber growth by DRG explants on CNS and PNS tissue substrata. Chick DRGs were seeded onto substrata as in Figure 1. Only ganglia directly attached to tissue sections were included in this analysis. The *ordinate* shows the percentage of those ganglia evaluated that grew minimally, moderately, or extensively (see Materials and Methods). Number of ganglia evaluated: sciatic nerve, $n = 86$; spinal cord, $n = 18$; optic nerve, $n = 63$.

Our data suggest that adhesive molecules within the ECM may be important determinants of nerve fiber growth in culture and *in vivo*.

Materials and Methods

Preparation of tissue substrata. Adult and embryonic Sprague-Dawley rats (Charles River), fertilized chicken eggs, and mature goldfish (supplied by Dr. R. Levine, McGill University) were used throughout this study. Adult rats were sacrificed by an overdose of chloral hydrate and perfused through the heart with PBS (0.1 M, pH 7.2). Segments of optic nerve, spinal cord, and sciatic nerve were excised. In all cases, sciatic nerves were desheathed and optic nerves and spinal cord freed of meninges. In experiments with injured PNS and CNS tissues, substrata were prepared from rats that had optic and sciatic nerve transections performed unilaterally under chloral hydrate anesthesia. Degenerating nerves were excised and used 5–10 d later. The tissue was then mounted, frozen in liquid nitrogen, and either stored at −70°C or cut into sections on a cryomicrotome. Sections were mounted onto round coverslips (15 mm in diameter) of glass or tissue culture plastic (Thermanox, Lux) that had been coated overnight with polylysine (1 mg/ml in borate buffer, pH 9). Prior to use, these substrata were sterilized by brief uv irradiation and washed extensively in culture medium.

Preparation of neuronal cultures. For most of our experiments, cultures of ganglionic explants from DRG or sympathetic ganglia were prepared from 9–12 d chick embryos by conventional methods (Carbonetto and Fambrough, 1978). Cells were cultured either in Dulbecco's Minimum Essential Medium (DMEM) plus 10% fetal calf serum, cy-

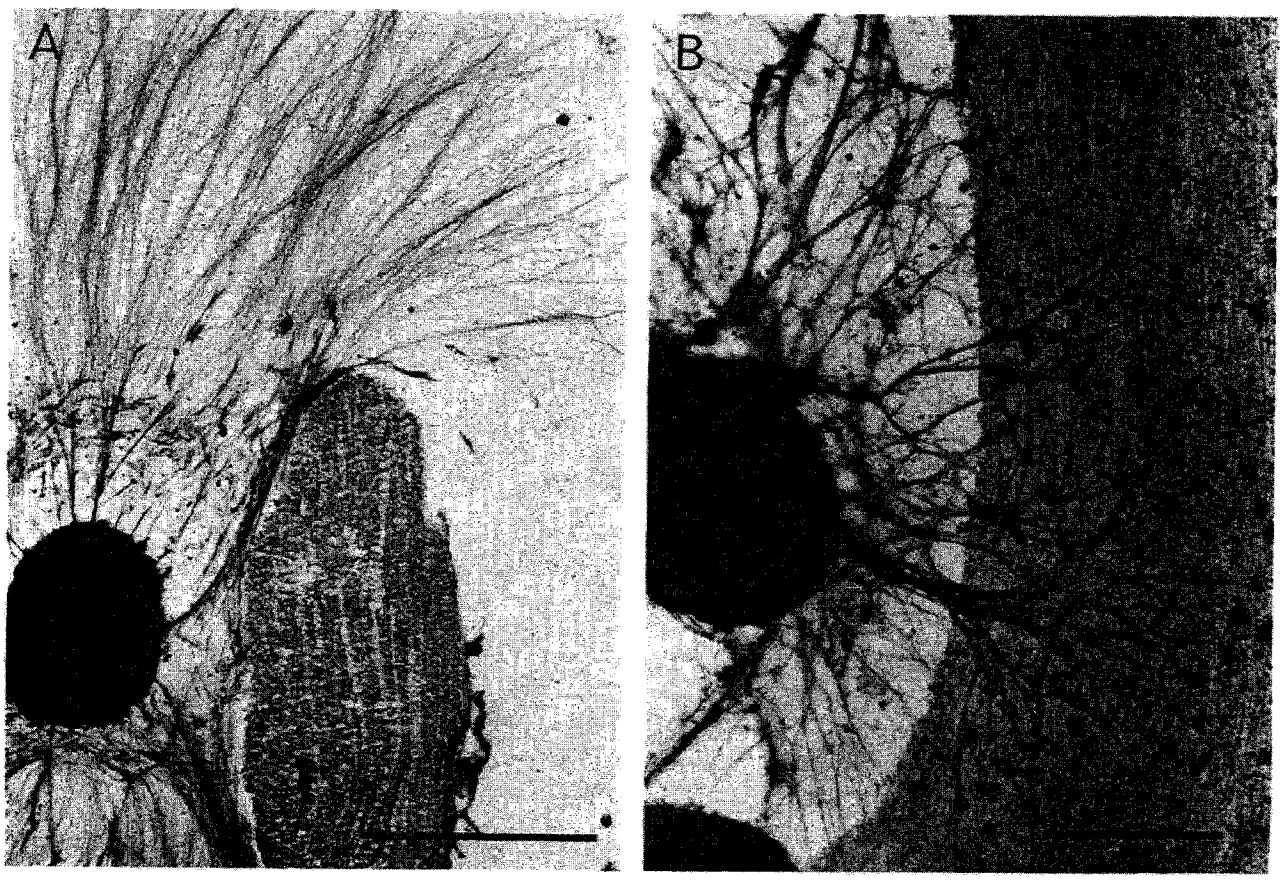

Figure 3. Nerve fiber growth onto sciatic nerve and optic nerve tissue from distant ganglia. Ganglia generally grew extensively on the polylysine-coated plastic, producing a halo of fine nerve fibers that by 2 d in culture were essentially free of non-neuronal cells. *A,* These fibers approached a section of optic nerve and grew along the periphery of the section but did not grow onto the section. Calibration bar, 0.5 mm. *B,* Ganglia distant from sections of sciatic nerve grew extensively on the culture plastic and onto the tissue substrata. Calibration bar, 0.25 mm.

tosine arabinoside (20 μM), and gentamicin or in defined medium (Bottenstein and Sato, 1978). Unless noted, all media contained NGF (50 ng/ml). Cultures of dissociated cells enriched for neurons (>80% neurons) were prepared by briefly treating ganglia with trypsin, triturating them, and plating the dissociated cells on petri dishes coated with serum. During this preplating step non-neuronal cells (fibroblasts and glia) preferentially adhere to the culture substratum and the cells enriched for neurons can be harvested by simply shaking the dish and aspirating the medium. PC12 cells were maintained as described by Greene and Tischler (1976) and treated with NGF for approximately 6 d before seeding onto tissue substrata. Ganglionic explants or dissociated cells (2×10^5 cells/ml) were seeded onto coverslips coated with tissue sections (100–200 μl/coverslip) placed in 35 mm culture dishes. Cultures were fed the following day with fresh medium and maintained for 2–5 d to allow nerve fiber growth.

Quantification of neuron–substratum adhesion and nerve fiber growth. Neuronal somata or nerve fibers from ganglionic explants located directly on tissue sections could not be reliably visualized in living cultures. To quantify neuronal adhesion and nerve fiber growth, the cultures were washed with DMEM, fixed with paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.2), rinsed in PBS, and stained with Coomassie Blue (0.1% in 10% acetic acid, 25% propanol) for 4 min. Following staining, nerve fibers were easily visible on tissue sections, as were any non-neuronal cells.

Neuron substratum adhesion was quantified by seeding DRG cells enriched for neurons and counting the somata that attached to tissue sections. The cell density was determined by counting the total number of cells attached per substratum and measuring the surface area of the tissue substrata with an IBAS image analyzer (Zeiss).

Nerve fiber growth from ganglionic explants was separated into 2 categories: (1) growth from ganglia attached to and growing directly on top of tissue substrata or (2) growth from ganglia attached to the culture plastic at some distance from the tissue and subsequently growing onto it. In the first category, nerve fibers that grew a distance of about 1 diameter of the ganglion were considered to be growing minimally. Less than this distance was considered negligible growth, and more than this was moderate (approximately 2 diameters of the ganglion) or extensive

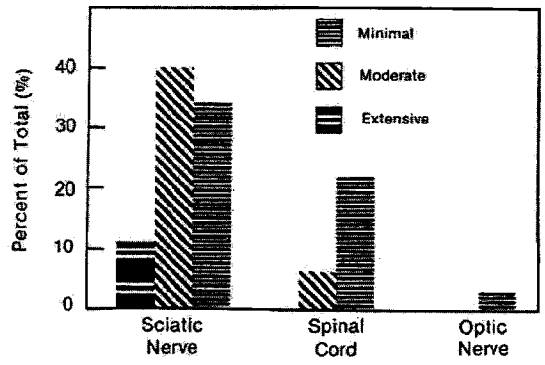

Figure 4. Nerve fiber growth onto CNS and PNS tissue substrata from DRGs distant from the tissue. Cultures were prepared as in Figure 3. Only ganglia not in contact with tissue sections and clearly growing fibers were included in this analysis. The *ordinate* gives the percentage of ganglia growing on the culture plastic that grew minimally, moderately, or extensively on the tissue. Number of ganglia: sciatic nerve, $n = 65$; spinal cord, $n = 18$; optic nerve, $n = 36$.

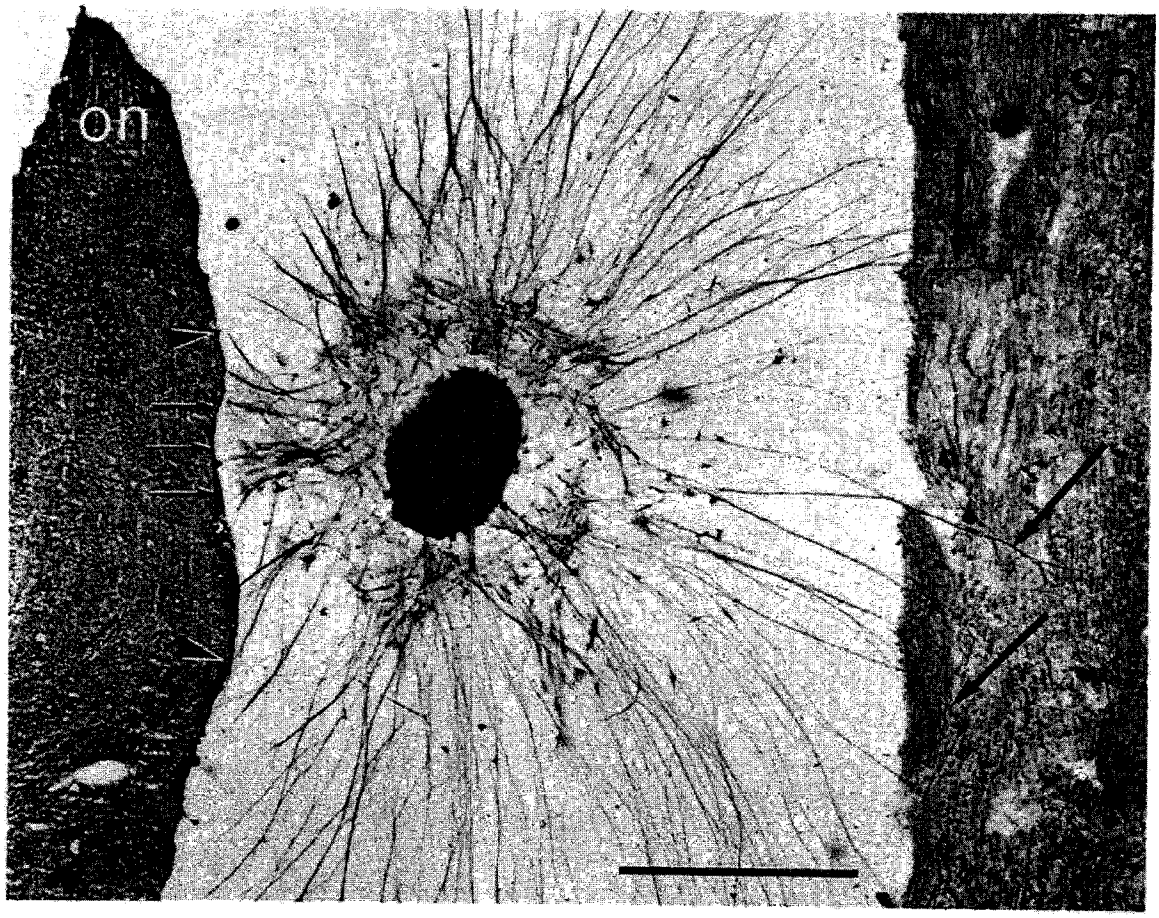

Figure 5. Nerve fiber growth on sciatic nerve and optic nerve sections within the same culture. For these experiments, tissue sections of sciatic nerve and optic nerve were placed side by side and ganglia seeded between the sections. The ganglion grew extensively up to the optic nerve section but not onto it. Fibers can be seen growing around the section (*arrowheads*), while others may have stopped growing altogether (*small arrows*). Growth of the same ganglion to the somewhat more distant sciatic nerve section resulted in readily observable growth of the fibers onto the tissue (*arrows*). Calibration bar, 0.5 mm.

growth (more than 2 diameters of the ganglion). In the second category, growth onto the tissue substrata from distant ganglia was quantified as minimal (<10 fibers on the tissue), moderate (10–50 fibers on the tissue), or extensive (>50 fibers on the tissue). For culture of dissociated neurons, individual neurons and all aggregates extending nerve fibers on the tissue were summed, and neuronal growth was expressed as a percentage of the total number of aggregates attached to the tissue substrata. Data were analyzed for statistical significance by comparing means directly for the least significant difference (Steel and Torrie, 1980).

Immunocytochemistry. For immunocytochemical localization of extracellular matrix components (laminin, fibronectin, heparan sulfate proteoglycan), rats were perfused with 4% paraformaldehyde and the tissues of interest excised. The tissue was sectioned (10 μm) on a cryostat as in preparing tissue substrata. Sections were then preincubated for 30 min in 3% albumin in PBS and subsequently incubated overnight with affinity-purified antibodies to horse serum fibronectin, and antisera to EHS laminin and to heparan sulfate proteoglycan (a gift from Dr. John Hassel, NIH). The antibodies were thoroughly washed free from the tissue, the sections incubated with FITC-conjugated goat anti-rabbit IgG (Cappel), thoroughly washed again in PBS, and mounted in Tris-buffered glycerol (pH 9).

Results

Nerve fiber growth by ganglionic neurons on adult rat sciatic nerve, optic nerve, and spinal cord

Explants of DRG or sympathetic ganglia attached to substrata of sciatic nerve, optic nerve, or spinal cord from adult rats. Once attached to sciatic nerve sections, the ganglia extended a halo of nerve fibers onto the tissue and surrounding culture plastic (Figs. 1*B*, 2). In contrast, ganglia attached to optic nerve or spinal cord substrata grew poorly, if at all, on the CNS tissue (Fig. 1*A*). For example, 66% (n = 68) of the ganglia on sciatic nerve grew moderately compared with none on optic nerve (n = 63) (Fig. 2).

Ganglionic explants contain non-neuronal cells (Schwann cells and fibroblasts) as well as neurons. These non-neuronal cells are known to stimulate nerve fiber outgrowth from neurons attached to them (Ludueña, 1973), as well as from neurons placed on substrata coated with materials they produce (Helfand et al., 1976; Collins, 1978; Hawrot, 1980). This raises the possibility that the tissue sections are acting indirectly, through ganglionic non-neuronal cells, to affect nerve fiber growth. To examine this possibility we analyzed the growth of ganglionic explants attached to the culture plastic distant from the tissue sections. In these experiments, cytosine arabinoside in the medium inhibited cell division and the halo of nerve fibers extending from ganglia was largely free of non-neuronal cells (Fig. 3) (Wood, 1976). Of the ganglia growing to contact sciatic nerve, more than 72% (n = 65) grew at least minimally onto the tissue substratum (Figs. 3*B*, 4). In comparison, 98% of the ganglia (n = 36) that had fibers contacting sections of optic nerve and 78% of those contacting spinal cord (n = 18) showed no growth onto the tissue. In several instances nerve fibers were found to

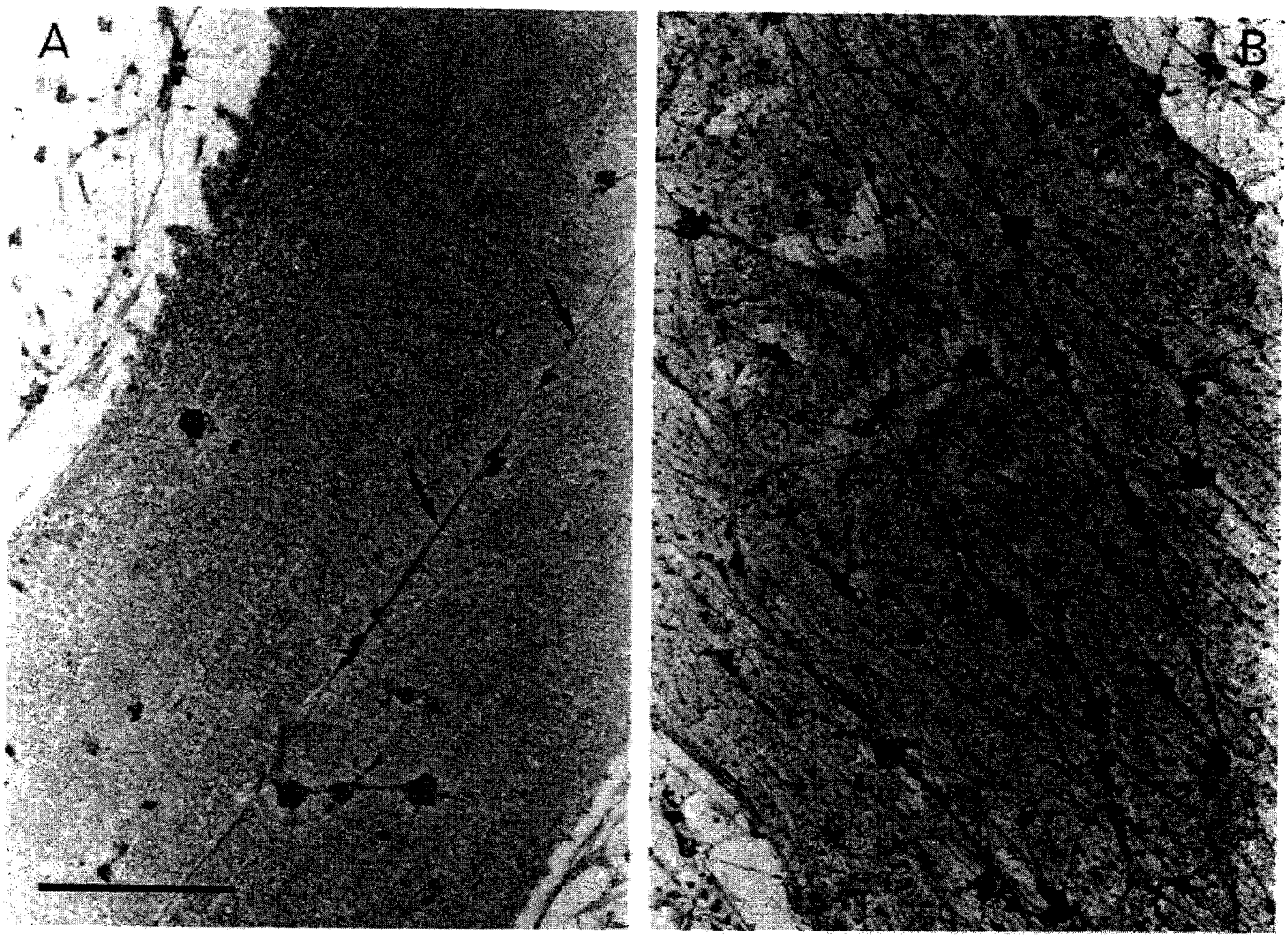

Figure 6. Dissociated DRG neurons on sections of optic nerve and sciatic nerve. *A*, Sections of optic nerve had few cells attached, and these showed little fiber growth. Interestingly, there was frequent attachment and fiber growth along the periphery of the tissue, which, in this culture, appears as a "septum" between 2 adjacent sections (*arrows*). Calibration bar, 250 μm. *B*, On sections of sciatic nerve, dissociated cells attached, and extended nerve fibers readily. Same magnification as in *A*.

grow on the periphery of the CNS tissue (Fig. 3*A*), as if growing on the residual connective tissue sheath. Culturing DRG cells in serum-free medium (Bottenstein and Sato, 1978) yielded the same results: growth on PNS (sciatic nerve) but not on CNS (optic nerve) substrata. Thus, neither non-neuronal cells or their products nor serum components such as fibronectin appear to modify tissue substrata such that growth results only on PNS substrata.

The pattern of nerve fiber growth on the culture coverslip but not on the optic nerve substratum nearby suggests a local effect rather than one produced by factors diffusing some distance from the tissue. For example, outgrowth from ganglionic areas adjacent to sciatic nerve sections was not obviously more extensive than from regions of the ganglia away from the section. Similarly, fibers from ganglia near optic nerve grew to contact the tissue before growing around it (Fig. 3*A*). To determine more directly whether a diffusible agent(s) might be responsible for the growth of DRG explants on sciatic nerve but not CNS, ganglia were cultured on coverslips between sections of optic or sciatic nerve. Among those that grew to contact both PNS and CNS substrata (n = 23), 70% (8 minimal growth, 8 moderate growth) showed a marked preference for sciatic nerve (Fig. 5). In no case was there any growth on optic nerve. In a smaller sample where ganglia were given a "choice" of spinal cord or sciatic nerve, 75% of the cases showed growth on sciatic nerve with none on spinal cord (n = 4).

The growth of these distant ganglia whose nerve fibers are free of non-neuronal cells onto sciatic but not optic nerve is consistent with the hypothesis that the fibers are responding to the tissue sections themselves and not to factors produced by non-neuronal cells (see Discussion). Moreover, the pattern of outgrowth of ganglia placed between optic and sciatic tissues suggests that this effect is not mediated by a diffusible substance(s) but by direct neuron–substratum contact.

Dissociated neurons: adhesion to and growth on sciatic nerve and optic nerve substrata

Ganglionic explants are covered by a capsule of connective tissue that is difficult to remove and probably restricts outgrowth from regions of ganglia or inhibits growth entirely (approximately 10–25% of our ganglionic explants failed to extend processes on any substratum). By preparing enriched cultures of DRG and sympathetic neurons it was possible to avoid the limitations resulting from the encapsulating connective tissue and the variability of fiber outgrowth from ganglia. Dissociated DRG neurons behaved qualitatively the same as explanted gan-

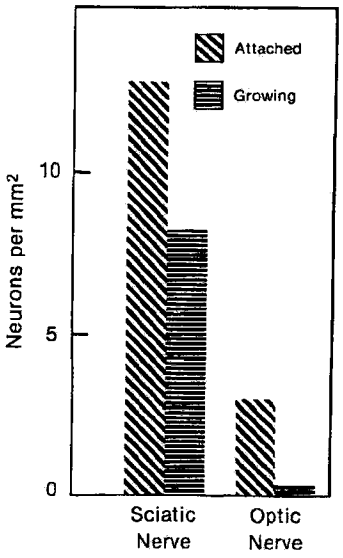

Figure 7. Quantification of attachment and growth of dissociated DRG neurons on sections of optic and sciatic nerves. The *ordinate* gives the mean density of cells and cell-aggregates attached to the sections and extending nerve fibers onto them.

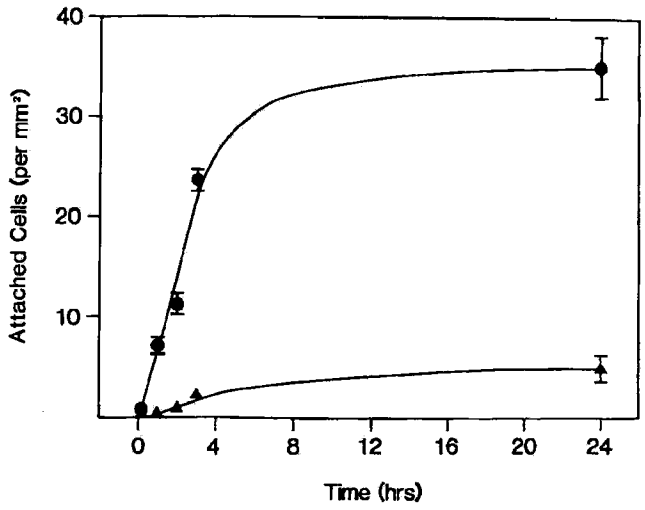

Figure 8. Kinetics of DRG cell attachment to substrata of optic nerve and sciatic nerve. DRG cells enriched for neurons were seeded onto substrata of sciatic nerve (●) or optic nerve (▲). At times indicated along the *abscissa* cultures were removed from the incubator, washed gently with HBSS, and fixed. The number of attached cells was quantified by light-microscopic observation (see Materials and Methods). The error bars are shown where they are larger than the symbol and represent the SEM. The number of attached cells counted ranged from 8/culture (optic nerve at 10 min) to 629/culture (sciatic nerve at 24 hr).

glia, growing on sciatic nerve substrata but not on optic nerve. Although seeded as single cells, the neurons often aggregated into small clumps on the tissue substrata (Fig. 6). By 2 d in culture there were fewer cells attached to optic nerve substrata than to sciatic nerve (Figs. 6, 7), and the outgrowth of nerve fibers was much greater on sciatic nerve than on optic nerve (8.7 aggregates/mm² on sciatic nerve vs 0.4 aggregates/mm² on optic nerve). Often the pattern of growth in these cultures appeared to follow the longitudinal axis of the sciatic nerve (Fig. 6*B*).

The reduced number of cells attached to the optic nerve sections could have arisen either from diminished adhesion or enhanced detachment of neurons from optic nerve. Cultured cells are well known to adhere selectively to culture substrata and other cells. Neurons in culture will also detach selectively from other cells (Ruffolo et al., 1978). The kinetics of attachment of DRG cells to sciatic nerve and optic nerve substrata (Fig. 8) indicated that even by 1 hr attachment to sciatic nerve was significantly greater than to optic nerve and this attachment increased monotonically over the next 4 hr. There was no evidence for selective detachment of cells from optic nerve that could be responsible for the 7-fold difference between the number of cells attached to sciatic nerve and to optic nerve.

Other sources of neurons and tissue substrata

It is well known that culture plastic coated with basic polyaminoacids such as polylysine or polyornithine facilitate neuron–substratum adhesion (Fig. 3) (Letourneau, 1975). However, polylysine would not be expected to serve as a substratum for nerve fiber growth *in vivo* since it is not likely to be present on the surfaces of neural cells. Nevertheless, the robust effects of polylysine-coated substrata in supporting nerve fiber growth caution that molecules only partially related or entirely distinct from those operating *in vivo* may stimulate fiber growth in culture. With this in mind, we examined the effects of CNS and PNS tissues from a variety of sources to correlate their known efficacy at supporting nerve fiber growth *in vivo* with that in culture. Like adult optic nerve, spinal cord from adult rats supported dramatically less nerve fiber growth from dissociated neurons than did sciatic nerve (Figs. 9*D*, 10), thus confirming results obtained with whole ganglia (see Fig. 1*A*). However, substrata of spinal cord tissue from 14 and 16 d embryonic rats supported extensive fiber growth in culture, approaching that of sciatic nerve (Figs. 9*C*, 10). On substrata of optic nerve from mature goldfish substantially less nerve fiber growth was observed than on either sciatic nerve or embryonic spinal cord sections but there was significantly more than on adult rat spinal cord (Figs. 9*B*, 10). Substrata of degenerating rat sciatic nerve (Fig. 9*A*) were also effective for fiber growth but not obviously more so than normal sciatic nerve (Fig. 6*B*). In conclusion, several substrata from nervous tissue (sciatic nerve, embryonic spinal cord, goldfish optic nerve) that support nerve fiber growth *in vivo* also support significant growth of DRG neurons in culture. Other substrata (adult rat optic nerve and spinal cord) that support little fiber growth *in vivo* support correspondingly little growth in culture. This correlation of activity *in vivo* with that in culture supports the notion that molecules residing in tissue substrata contribute to the control of nerve fiber growth *in vivo*. Moreover, the growth of neurons on rat sciatic nerve but not on optic is not restricted to DRG neurons. Similar results were found with explants of embryonic chick sympathetic ganglia, dissociated sympathetic neurons, and PC12 cells (data not shown).

Immunocytochemical localization of extracellular matrix components in PNS and CNS substrata

Regenerating nerve fibers *in vivo* are often found in close apposition to basement membranes (Ide et al., 1983). Among the molecular constituents of basement membranes, fibronectin (Akers et al., 1981; Carbonetto et al., 1982; Rogers et al., 1983), Type IV collagen (Carbonetto et al., 1983), laminin (Baron van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al.,

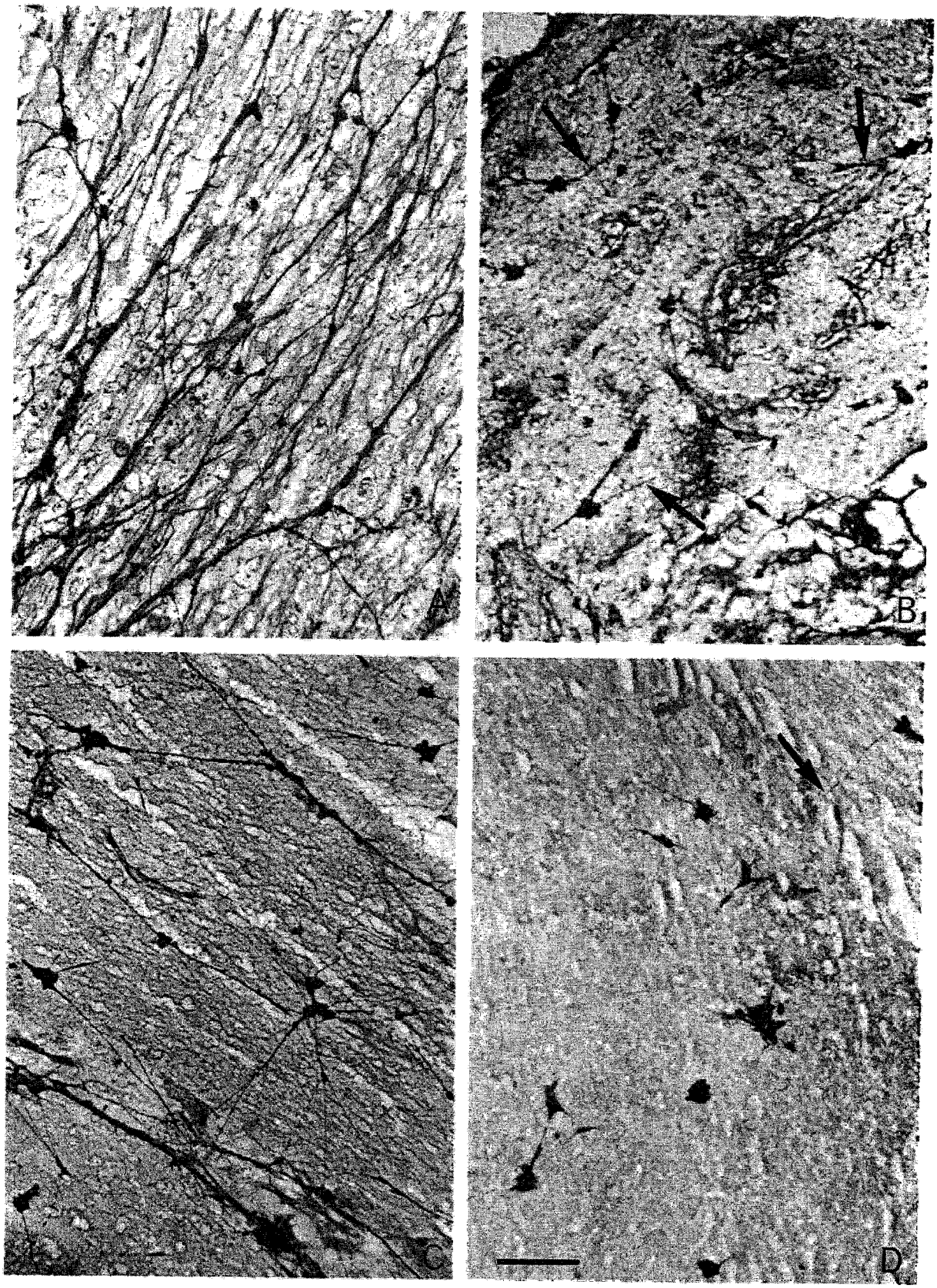

Figure 9. Nerve fiber growth on various neural tissue substrata. *A*, Extensive nerve fiber outgrowth occurred on sections of degenerating rat sciatic nerve, as it did on normal nerve (see Fig. 6*B*). Note that in both cases the general pattern of nerve fiber outgrowth followed the long axis of the nerve. *B*, Nerve fiber outgrowth (*arrows*) was also observed on sections of goldfish optic nerve, although it was much less extensive than on rat PNS tissue. *C*, Dissociated DRG neurons also readily extended long, thin processes on sections of embryonic (16 d) rat spinal cord. *D*, In comparison, fiber outgrowth was virtually absent on adult rat spinal cord, except in rare instances (*arrow*). Scale bar in *D* represents 100 μm and applies to all micrographs.

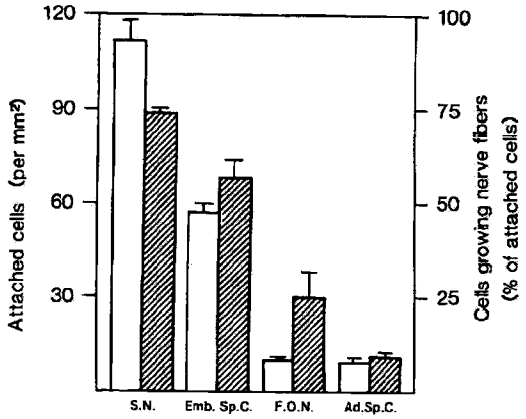

Figure 10. Quantification of fiber growth on various neural tissue substrata. Dissociated DRG neurons were seeded onto substrata (3 cultures each) of the following tissues: rat sciatic nerve (*S.N.*), spinal cord from 14 d embryonic rats (*Emb.Sp.C.*), goldfish optic nerve (*F.O.N.*), and adult rat spinal cord (*Ad.Sp.C.*). After approximately 2 d the cultures were fixed and the number of attached cells (*open bars, left ordinate*) and neurons growing nerve fibers were counted (*shaded bars, right ordinate*). The numbers of attached cells counted ranged from 220 to 738/culture. The number of neurons growing nerve fibers counted ranged from 49 to 528/culture. Sciatic nerve and embryonic spinal cord ($p < 0.001$), as well as fish optic nerve ($p < 0.05$), were significantly better substrata for nerve fiber growth than adult spinal cord sections.

1983; Smalheiser et al., 1984; Lander et al., 1985), and heparan sulfate proteoglycan (Culp et al., 1980) have all been implicated in neuron substratum adhesion and/or nerve fiber growth. Since our studies indicate that neurons adhere less well to CNS than to PNS tissues, we sought to localize immunocytochemically some of these ECM components in our tissue substrata (Fig. 11).

Immunoreactivities for laminin, heparan sulfate proteoglycan, and fibronectin were readily detected in normal and degenerating sciatic nerves. Laminin and heparan sulfate proteoglycan were clearly restricted to a thin layer in the endoneurium, most probably the basement membrane. Fibronectin appeared more diffuse in the endoneurium and perineurium. None of these ECM components could be found within the optic nerve or spinal cord of adult rats, except in the external connective tissue layer and in the basement membranes of blood vessels. Interestingly, in 14 and 16 d embryonic spinal cord, striking differences were observed in the staining patterns of these various antigens. Laminin was present almost throughout the tissue surrounding neuroepithelial cells, whereas fibronectin and heparan sulfate proteoglycan were largely restricted to the connective tissue and vascular components, as in adult CNS tissue. Laminin was also detectable in fish optic nerve, localized in septa partitioning nerve bundles, but more diffusely than in the basal lamina of peripheral nerve. Low but detectable levels of fluorescence for fibronectin and heparan sulfate proteoglycan were also found in punctate and fibrillar distributions in the fish optic nerve. Identical localizations for all these antigens were found in tissue sections used as culture substrata for 2 d, indicating that these molecules persist in culture and are likely available to interact with nerve fibers.

Discussion

The purpose of our studies was to explore *in vitro* the properties of neural tissue with regard to its ability to support nerve regeneration. The approach we have taken, using sections of neural tissues as culture substrata, was prompted by 2 sets of experiments. First, Ide and coworkers (1983) reported that freeze-thawed peripheral nerves grafted into the CNS of adult rats supported some nerve fiber growth. Second, Schwab and Thoenen (1985) described the first system of cultured sciatic and optic nerve wherein axons from cultured neurons grew preferentially into explants of the PNS tissue. Indeed, our work should be viewed as an extension of that of Schwab and Thoenen (1985), as well as that of Sandrock and Matthew (1985), who reported a culture system apparently identical to the one we have described here. This culture system uses sections of neural tissue, rather than organ-cultured optic and sciatic nerves (Schwab and Thoenen, 1985), as substrata for embryonic neurons. This method allows direct visualization of nerve fiber growth on the tissues at the light-microscopic level without resort to light- or electron-microscopic analysis of sectioned material. Substantial nerve fiber growth can be seen in 1–2 d, rather than several weeks, and the fiber growth is easily quantified following fixation and staining with Coomassie Blue (Materials and Methods). In addition, the substrata of tissue sections on glass or plastic coverslips are easy to prepare and neurons can be given a choice of CNS or PNS substrata (Fig. 5) without constructing 3-chamber culture vessels (Campenot, 1977; Schwab and Thoenen, 1985).

Our data are in substantial agreement with those of Schwab and Thoenen (1985) and Sandrock and Matthew (1985). We find that nerve fiber growth is significantly greater on PNS tissue than on adult CNS tissue. Additionally, we show that several neural tissues that support nerve fiber growth *in vivo* (normal and degenerating rat sciatic nerve, embryonic rat spinal cord, fish optic nerve) also support growth of cultured neurons. Neural tissues that do not support fiber growth *in vivo* (adult rat optic nerve and spinal cord) also support little growth in culture. The observed difference between CNS and PNS substrata does not appear to be due to a diffusible agent(s) released from the tissue (Schwab and Thoenen, 1985) but rather to a local effect of the tissue. We believe this local effect requires contact of the neurons with the tissue and is manifest in the greater adhesion of DRG cells to sciatic nerve than to optic nerve.

It is important to consider whether neurons interact directly with the tissue substrata and not with other cultured cells, their products, or the constituents of the culture medium. In our studies, ganglionic explants produce halos of axons essentially devoid of non-neuronal cells, which nevertheless grow onto sections of sciatic nerve but not optic nerve. This suggests that sciatic nerve sections do not act through non-neuronal cells to stimulate nerve fiber growth. Furthermore, growth of neurons in serum-free medium suggests that fibronectin or other serum components that can mediate fiber growth are not binding to sciatic nerve sections so as to enhance fiber growth. However, we cannot eliminate the possibility that the cells themselves produce some molecules that attach preferentially to sciatic nerve to stimulate growth. Even though we see differential adhesion to sciatic and optic substrata within 1–2 hr, the cells might, in that brief period, be modifying the substrata.

The simplest explanation for the growth of nerve fibers on PNS, but not CNS, substrata is that a single, positive effector of growth is present in sciatic nerve but not in optic nerve. Alternatively, there may be a mix of negative (Schwab and Thoenen, 1985) and positive effectors in optic nerve and sciatic nerve, respectively. Sandrock and Matthew (1985) reported that a monoclonal antibody that binds to a heparan sulfate proteo-

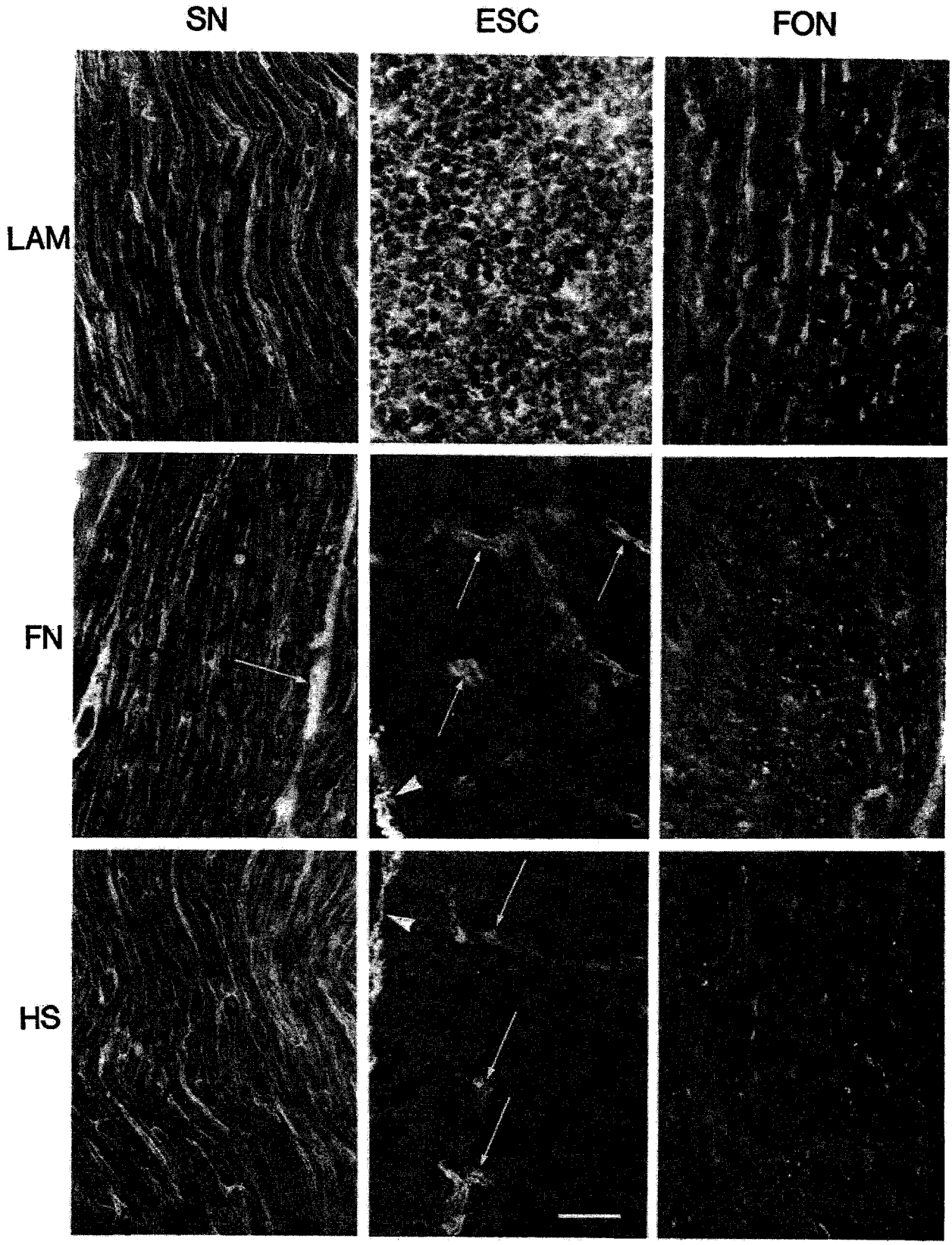

Figure 11. Immunocytochemistry of laminin (*LAM*), fibronectin (*FN*), and heparan sulfate proteoglycan (*HS*) in longitudinal sections of adult rat sciatic nerve (*SN*), rat embryonic (14.5 d) spinal cord (*ESC*), and mature goldfish optic nerve (*FON*). LAM, FN, and HS immunoreactivities are all found in sciatic nerve sections, located in the endoneurial extracellular matrix, fluorescence for FN being somewhat stronger in the perineurium (*arrow* in FN/SN). Strong immunoreactivity for LAM is also found around most cells of the rat embryonic spinal cord (LAM/ESC) and is present as well in the fish optic nerve (LAM/FON), although its cellular localization is more diffuse. In contrast, FN and HS immunoreactivities in embryonic spinal cord (ESC) are restricted to connective tissue (*arrowheads*) and blood vessels (*arrows*), as in the adult (not shown). The fish optic nerve (FON) displays only low levels of FN and HS immunoreactivities, in punctate and fibrillar forms. Scale bar in HS/ESC represents 50 μm and applies to all micrographs.

glycan in the ECM of sciatic nerve inhibits nerve fiber growth on sciatic nerve sections. If this antibody inhibits the activity of a single species of molecule, then there is only one positive effector. However, the mode of action of this antibody appears to involve binding to a heparan sulfate proteoglycan–laminin complex in an as yet undescribed manner. Thus, the number and identity of the positive effector(s) and of the negative one(s), if any, remain to be detailed.

In keeping with observations made by others (Schachner et al., 1978; Paetau, 1980; Cornbrooks et al., 1983; Laurie et al., 1983), laminin, fibronectin, and heparan sulfate proteoglycan can be immunocytochemically identified in the endoneurium of sciatic nerve. These antigens are much less prominent in adult rat CNS. The distribution of fibronectin and heparan sulfate proteoglycan was essentially the same in the embryonic and adult rat tissues, i.e., absent throughout the brain and restricted to vascular and connective tissue basement membranes. In contrast, a high level of laminin immunoreactivity is found around most cells of the spinal cord of rat embryos (14–16 d). Similarly, laminin is readily visualized in goldfish optic nerve (Hopkins et al., 1985; Liesi, 1985b), while fibronectin and heparan sulfate proteoglycan are present in low but detectable levels. Thus, laminin is demonstrable in all of the tissue substrata that support nerve fiber growth.

The laminin distribution we found in the embryonic rat spinal cord and goldfish optic nerve is very similar to that reported by Liesi for embryonic rat brain (1985a) and goldfish optic nerve (1985b). Others have failed to detect laminin immunohistochemically in embryonic rat brain and spinal cord (Bignami et al., 1984) or in the developing chick CNS (Rogers et al., 1986). These discrepancies may result from differences in fixation procedures, antisera, embryonic age, etc. Alternatively, our antisera to laminin may be cross-reacting with other molecules. ECM components are well known to copurify (Bayne et al., 1984), which presents difficulties in producing monospecific antisera to them. It is noteworthy that we have found the same distribution of laminin with 2 different antisera, one commercial (Bethesda Research Labs) and one produced by ourselves. The latter is highly specific for laminin and does not cross-react with fibronectin or collagen (S. Carbonetto, unpublished observations). We are presently producing monoclonal antibodies to laminin, which should help considerably in resolving this important point concerning the distribution of laminin in embryonic CNS.

Our studies have focused on the adhesive proteins of the ECM (laminin, fibronectin, heparan sulfate proteoglycan). Another class of adhesive molecules (cell adhesion molecules, or CAMs) also affects nerve fiber growth (Rutishauser et al., 1978) and could be involved in the growth of nerve fibers on our nerve tissue substrata. However, we see little growth on CNS tissue, which, like PNS, has NCAM and NgCAM (reviewed by Edelman, 1984). To a first approximation, this suggests that CAMs are not involved in the differential growth of fibers on these tissues.

The amounts, forms, and distribution of adhesive proteins in the ECM of the nervous system are important determinants of a neuronal microenvironment's ability to support nerve fiber growth, as shown by a number of culture experiments and by correlative studies *in vivo* (discussed above). However, it is likely that regeneration of nerve fibers *in vivo* is multifactorial. Consider that some CNS neurons fail to regenerate into PNS grafts in adult rats (Aguayo, 1985). The presence in PNS, but not in CNS, sections of ECM molecules mediating neuron–substratum adhesion suggests to us that the failure of nerve fiber growth within the adult CNS microenvironment results, in part, from its unsuitability as an adhesive substratum for nerve fiber growth *in vivo*.

References

Aguayo, A. J. (1985) Axonal regeneration from injured neurons in the adult mammalian central nervous system. In *Synaptic Plasticity,* C. W. Cotman, ed., Guilford, New York.

Aguayo, A. J., R. Dickson, J. Trecarten, and M. Attiwell (1978) Ensheathment and myelination of regenerating PNS fibers by transplanted optic nerve glia. Neurosci. Lett. *9:* 97–104.

Aguayo, A. J., S. David, P. Richardson, and G. M. Bray (1979) Axonal elongation in peripheral and central nervous system transplants. Adv. Cell Neurobiol. *3:* 215–234.

Akers, R. M., D. F. Mosher, and J. E. Lilien (1981) Promotion of retinal neurite outgrowth by substratum-bound fibronectin. Dev. Biol. *86:* 179–188.

Barde, Y. A., D. Edgar, and H. Thoenen (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J. *1:* 549–553.

Baron van Evercooren, A., H. K. Kleinman, S. Ohno, P. Marangos, J. P. Schwartz, and M. E. Dubois-Dalq (1982) Nerve growth factor, laminin and fibronectin promote neurite growth in human fetal sensory ganglion cultures. J. Neurosci. Res. *8:* 179–194.

Bayne, E. K., M. J. Anderson, and D. M. Fambrough (1984) Extracellular matrix organization in developing muscle: Correlation with acetylcholine receptor aggregates. J. Cell Biol. *99:* 1486–1501.

Berg, D. K. (1984) New neuronal growth factors. Annu. Rev. Neurosci. *7:* 149–170.

Bignami, A., N. H. Chi, and D. Dahl (1984) First appearance of laminin in peripheral nerve, cerebral blood vessels and skeletal muscle. Int. J. Dev. Neurosci. *2:* 367–376.

Bottenstein, J. E., and G. H. Sato (1978) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA *76:* 514–517.

Campenot, R. B. (1977) Local control of neurite development by nerve growth factor. Proc. Natl. Acad. Sci. USA *74:* 4516–4519.

Carbonetto, S., and D. M. Fambrough (1978) Synthesis, insertion in the plasma membrane and turnover of α-bungarotoxin receptors in chick sympathetic neurons. J. Cell Biol. *81:* 555–569.

Carbonetto, S., M. M. Gruver, and D. C. Turner (1982) Nerve fiber growth on defined hydrogel substrates. Science *216:* 897–899.

Carbonetto, S., M. M. Gruver, and D. C. Turner (1983) Nerve fiber growth in culture on fibronectin, collagen and glycosaminoglycan substrates. J. Neurosci. *3:* 2324–2335.

Collins, F. (1978) Induction of neurite outgrowth by a conditioned medium factor bound to culture substratum. Proc. Natl. Acad. Sci. USA *75:* 5210–5213.

Cornbrooks, C. J., D. J. Carey, J. A. McDonald, R. Timpl, and R. P. Bunge (1983) In vivo and in vitro observations on laminin production by Schwann cells. Proc. Natl. Acad. Sci. USA *80:* 3850–3854.

Culp, L. A., R. Ansbacher, and C. Domen (1980) Adhesion sites of neural tumor cells: Biochemical composition. Biochemistry *19:* 5899–5907.

David, S., and A. J. Aguayo (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury. Science *214:* 931–933.

Davis, G. E., E. Engvall, and S. Varon (1985) Isolation and characterization of rat schwannoma neurite-promoting factor: Evidence that the factor contains laminin. J. Neurosci. *5:* 2662–2671.

DeGeorge, J. J., N. Slepecky, and S. Carbonetto (1985) Concanavalin A stimulates neuron–substratum adhesion and nerve fiber outgrowth in culture. Dev. Biol. *111:* 335–351.

Edelman, G. M. (1984) Modulation of cell adhesion during induction, histogenesis, and perinatal development of the nervous system. Annu. Rev. Neurosci. *7:* 339–377.

Edgar, D., R. Timpl, and H. Thoenen (1984) The heparin binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO J. *3:* 1463–1468.

Greene, L. A., and E. M. Shooter (1980) The nerve growth factor: Biochemistry, synthesis and mechanism of action. Annu. Rev. Neurosci. *3:* 353–402.

Greene, L. A., and A. S. Tischler (1976) Establishment of a noradrenergic clonal cell line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA *73:* 2424–2428.

Hawrot, E. (1980) Cultured sympathetic neurons: Effects of cell-derived and synthetic substrata on survival and development. Dev. Biol. *74:* 136–151.

Helfand, S. L., G. A. Smith, and N. K. Wessells (1976) Survival and development in culture of dissociated parasympathetic neurons from ciliary ganglia. Dev. Biol. *50:* 541–547.

Hopkins, J. M., S. Ford-Holevinski, J. P. McCoy, and B. W. Agranoff (1985) Laminin and optic nerve regeneration in the goldfish. J. Neurosci. *5:* 3030–3038.

Ide, C., K. Tohyama, R. Yokota, T. Nitatori, and S. Onodera (1983) Schwann cell basal lamina and nerve regeneration. Brain Res. *288:* 61–75.

Klebe, R. J., K. L. Bentley, and R. C. Schoen (1981) Adhesive substrates for fibronectin. J. Cell Physiol. *109:* 481–488.

Lander, A. D., D. K. Fujii, and L. F. Reichardt (1985) Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned media. Proc. Natl. Acad. Sci. USA *82:* 2183–2187.

Laurie, G. W., C. P. Leblond, and G. R. Martin (1983) Light microscopic immunolocalization of type IV collagen, laminin, heparan sulfate proteoglycan and fibronectin in the basement membranes of a variety of rat organs. Am. J. Anat. *167:* 71–82.

Letourneau, P. C. (1975) Cell to substratum adhesion and guidance of axonal elongation. Dev. Biol. *44:* 92–101.

Liesi, P. (1985a) Do neurons in the vertebrate CNS migrate on laminin? EMBO J. *4:* 1163–1170.

Liesi, P. (1985b) Laminin-immunoreactive glia distinguish regenerative adult CNS systems from non-regenerative ones. EMBO J. *4:* 2505–2511.

Ludueña, M. A. (1973) Nerve cell differentiation in vitro. Dev. Biol. *33:* 268–289.

Manthorpe, M., E. Engvall, E. Ruoshlahti, F. M. Longo, G. E. Davis, and S. Varon (1983) Laminin promotes neuritic regeneration from cultured peripheral and central neurons. J. Cell Biol. *97:* 1882–1890.

Nathaniel, E. J. H., and D. R. Nathaniel (1973) Regeneration of dorsal root fibers into the adult rat spinal cord. Exp. Neurol. *40:* 333–350.

Nathaniel, E. J. H., and D. C. Pease (1963) Regenerative changes in rat dorsal roots following Wallerian degeneration. J. Ultrastruct. Res. *9:* 533–549.

Paetau, A., K. Mellstrom, A. Vaheri, and M. Haltia (1980) Distribution of a major connective tissue protein, fibronectin, in normal and neoplastic human nervous tissue. Acta Neuropathol. *51:* 47–51.

Rauvala, H. (1984) Neurite outgrowth of neuroblastoma cells: Dependence on adhesion-surface, cell–surface interactions. J. Cell Biol. *98:* 1010–1016.

Richardson, P. M., U. M. McGuinness, and A. J. Aguayo (1980) Axons from CNS neurones regenerate into PNS grafts. Nature *284:* 264–265.

Rogers, S. L., P. C. Letourneau, S. L. Palm, J. McCarthy, and L. T. Furcht (1983) Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. *98:* 212–220.

Rogers, S. L., K. J. Edson, P. C. Letourneau, and S. C. McLoon (1986) Distribution of laminin in the developing peripheral nervous system of the chick. Dev. Biol. *113:* 429–435.

Ruffolo, R. R., G. S. Eisenbarth, J. M. Thompson, and M. Nirenberg (1978) Synapse turnover: A mechanism for acquiring synaptic specificity. Proc. Natl. Acad. Sci. USA *75:* 2281–2285.

Rutishauser, U., W. E. Gall, and G. M. Edelman (1978) Adhesion among neural cells of the chick embryo. IV. Role of the cell surface molecule CAM in the formation of neurite bundles in cultures of spinal ganglia. J. Cell Biol. *79:* 382–393.

Sandrock, A. W., and W. D. Matthew (1985) A bioassay for the analysis of neurite promoting factors in the extracellular matrix of rat peripheral nerve. Soc. Neurosci. Abstr. *11:* 592.

Schachner, M., G. Schoonmaker, and R. O. Hynes (1978) Cellular and subcellular localization of LETS protein in the nervous system. Brain Res. *158:* 149–158.

Schwab, M., and H. Thoenen (1985) Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. J. Neurosci. *5:* 2415–2423.

Smalheiser, N. R., S. M. Crain, and L. M. Reid (1984) Laminin as a substrate for retinal axons in vitro. Dev. Brain Res. *12:* 136–140.

Steel, R. G. D., and J. H. Torrie (1980) *Principles and Procedures of Statistics: A Biometrical Approach,* pp. 173–177, McGraw-Hill, New York.

Wood, P. M. (1976) Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. Brain Res. *115:* 361–375.

EXHIBIT C

Neurotrophic Factors and Neurologic Disease

DAVID M. HOLTZMAN, MD, *and* WILLIAM C. MOBLEY, MD, PhD, *San Francisco, California*

Discovered only 40 years ago, nerve growth factor is the prototypic neurotrophic factor. By binding to specific receptors on certain neurons in the peripheral nervous system and brain, nerve growth factor acts to enhance their survival, differentiation, and maintenance. In recent years, many additional neurotrophic factors have been discovered; some are structurally related to nerve growth factor while others are distinct from it. The robust actions of neurotrophic factors have suggested their use in preventing or lessening the dysfunction and death of neurons in neurologic disorders. We review the progress in defining neurotrophic factors and their receptors and in characterizing their actions. We also discuss some of the uses of neurotrophic factors in animal models of disease. Finally, we discuss how neurotrophic factors could be implicated in the pathogenesis of neurologic disorders.

(Holtzman DM, Mobley WC: Neurotrophic factors and neurologic disease, *In* Neurology—From Basics to Bedside [Special Issue]. West J Med 1994; 161:246-254)

Neurotrophic factors are polypeptides that exert their actions through binding and activating specific cell surface receptors. It is increasingly apparent that neurotrophic factors have an important role in the growth, development, and maintenance of neurons in both the central and peripheral nervous systems. Evidence accumulated over the past few years points to the existence of a number of classes of neurotrophic factors and documents their remarkable potency on responsive neurons (Table 1). These data are enhancing and accelerating efforts to apply neurotrophic factors to the treatment of human neurologic disease. In this article we briefly discuss the neurotrophic factors most likely to be used in clinical studies in the near future. The structure and functions of these molecules and their receptors are reviewed. Thereafter we consider data from three experiments in which neurotrophic factors have been used in animal models of neurologic disease and the mechanisms by which neurotrophic factors may be implicated in neurodegeneration and its treatment.

TABLE 1.—*Neurotrophic Factors—A Partial Listing*

Factor	*Abbreviation*
Neurotrophins	
Nerve growth factor	NGF
Brain-derived neurotrophic factor	BDNF
Neurotrophin 3	NT-3
Neurotrophin 4/5	NT-4/5
Ciliary neurotrophic factor	CNTF
Leukemia inhibitory factor	LIF
Insulin and the insulinlike growth factors	IGF-I IGF-II
Tumor growth factor-β family	TGFβ
Glial cell line-derived neurotrophic factor	GDNF
Fibroblast growth factors	FGF-1 FGF-2 FGF-5
Epidermal growth factor	EGF

Neurotrophic Factors

Nerve Growth Factor and the Neurotrophins

The neurotrophic factors in the neurotrophin gene family consist of nerve growth factor (NGF), the best characterized member, brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4/5. Nerve growth factor was discovered by Levi-Montalcini and Hamburger in the late 1940s and early 1950s. They were examining the effect a target has on its innervating neural center and discovered that a particular mouse sarcoma evoked dramatic hypertrophy of innervating dorsal root ganglia and sympathetic ganglia. The authors noted substantial deviations from the normal pattern of embryonic development in the extent of ganglion hyperplasia, the involvement of ganglia distant from the site of the sarcoma, and the bizarre, excessive innervation of various viscera.[1] These findings led to the hypothesis that the neoplastic cells released a soluble, diffusible agent that promoted the differentiation and growth of sympathetic and sensory neurons. This idea was supported in experiments in which the mouse sarcoma elicited the same effects when transplanted onto the chorioallantoic membrane of chick em-

From the Departments of Neurology (Drs Holtzman and Mobley) and Pediatrics and the Neuroscience Program (Dr Mobley), University of California, San Francisco, School of Medicine.

This work has been supported by grant Nos. NS24054, AG10672, and AG08938 from the National Institutes of Health and a grant from the March of Dimes Birth Defects Foundation.

Reprint requests to William C. Mobley, MD, PhD, Dept of Neurology, Rm M794, UCSF School of Medicine, San Francisco, CA 94143-0114.

ABBREVIATIONS USED IN TEXT

BDNF = brain-derived neurotrophic factor
cDNA = complementary DNA
CNTF = ciliary neurotrophic factor
GABA = γ-butyric acid
GDNF = glial cell line-derived neurotrophic factor
IGF-I, -II = insulinlike growth factors I, II
IL-6 = interleukin-6
LIF = leukemia inhibitory factor
mRNA = messenger RNA
NGF = nerve growth factor
NT-3, -4/5 = neurotrophins 3, 4/5

bryos, a location that allowed diffusion through the circulation but prevented direct contact between the sarcoma and developing tissue.[2] Levi-Montalcini and Hamburger called the diffusible agent nerve growth factor. They were later able to purify the material to homogeneity and discovered a rich source that permitted many subsequent investigations on its biology and chemistry.[3]

Nerve growth factor is a 118-amino acid polypeptide that acts on neurons in both the peripheral and central nervous systems. Responsive peripheral neurons include cells in the trigeminal sensory ganglion, dorsal root ganglia, and paravertebral sympathetic ganglia. Nerve growth factor also acts on adrenal chromaffin cells. In the central nervous system, two populations respond robustly to NGF. These are the cholinergic neurons of the basal forebrain and those of the caudate putamen.[3] Nerve growth factor activates responsive cells through binding to specific receptors on their surface.[4] One of these receptors is $p75^{NGFR}$. This glycosylated transmembrane protein binds NGF with low affinity. It appears to modulate NGF binding, but it is not yet clear how and to what extent it influences NGF signaling.[3] Another transmembrane glycoprotein, trkA, serves as a receptor for NGF. The intracellular domain of trkA encodes a tyrosine kinase (Figure 1). The binding of NGF to trkA causes it to dimerize; this activates its kinase and leads to autophosphorylation. Activating trkA kinase is necessary for transmitting the NGF signal.[4,5] Signal transduction is mediated through the phosphorylation of specific residues on trkA. These serve to bind and to activate other proteins, including phospholipase C-γ and phosphatidylinositol 3-kinase.[6,7] The signaling cascade produced by NGF is continued by a number of events, including the activation of other kinases and the generation of second messengers. The details of this cascade are only beginning to be discovered.

In both the peripheral and central nervous systems, NGF is produced in the target of innervating neurons.[3] There are clear examples where neurons have access to NGF mainly through contact with their targets. An interesting requirement imposed by this arrangement is the need to convey the NGF signal down the axon to the cell body. How this is achieved is not yet clear.[6] What is clear is that NGF signaling requires binding to trkA. In every case, NGF-responsive cells have been shown to bear trkA receptors. Thus, it appears that NGF produced in the target of developing and mature neurons binds to trkA on the neurites of these cells to initiate or maintain trophic relationships.

Brain-derived neurotrophic factor (BDNF) was discovered in 1982 as the culmination of a painstaking series of studies by Barde and colleagues.[8] It is a neurotrophic factor similar in structure to NGF. Indeed, the discovery of BDNF suggested the existence of a neurotrophin gene family. This neurotrophic factor is a 120-amino acid polypeptide that is 50% identical to NGF at the amino acid level. The sequence homology was used by several groups to isolate a third member of this family, neurotrophin 3 (NT-3),[9-13] and a fourth member, neurotrophin 4/5 (NT-4/5).[14-16] Cells in the peripheral nervous system that are responsive to BDNF include sensory neurons in the nodose ganglion and a subpopulation of dorsal root ganglia neurons. Brain-derived neurotrophic factor also acts on trigeminal mesencephalic neurons. Its targets in the central nervous system include retinal ganglion neurons, hippocampal neurons, basal forebrain cholinergic neurons, basal forebrain γ-aminobutyric acid (GABA)-ergic neurons, and GABAergic neurons of the ventral mesencephalon.[17,18] Recent studies have demonstrated that BDNF also acts on motoneurons.[19-22] This neurotrophic factor is found predominantly in the central nervous system, where it is most abundant in the adult. The highest levels of BDNF messenger RNA (mRNA) were found in the hippocampus, cortex, and cerebellum.[23] In situ hybridization histochemistry has localized BDNF mRNA to neurons in several brain regions. These include pyramidal, hilar, and dentate granule neurons of the hippocampus and neurons in the neocortex.[24] As with NGF, $p75^{NGFR}$

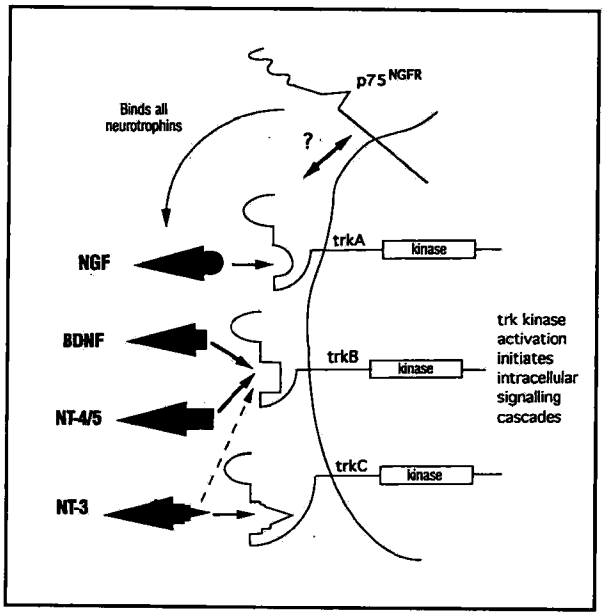

Figure 1.—The diagram illustrates the trk receptors and their specificities for the neurotrophins—nerve growth factor (**NGF**), brain-derived growth factor (**BDNF**), and neurotrophin (**NT**)-3 and NT-4/5. Ligand binding induces dimerization and autophosphorylation that activates signaling. The role of the low-affinity NGF receptor ($p75^{NGFR}$), which binds to all of the neurotrophins, is uncertain.

serves as a receptor for BDNF and binds this neurotrophic factor with low affinity. Another member of the trk gene family, trkB, is the signaling receptor for BDNF (Figure 1). Transcripts of trkB are found in many central nervous system regions, including hippocampus, basal forebrain, neocortex, ventral mesencephalon, and in spinal cord motoneurons.[6,18,24,25] There is an important distinction with respect to trkA and trkB: there are trkB transcripts that encode truncated proteins in which the tyrosine kinase domain is missing.[6] This finding raises the possibility that not all BDNF binding would lead to signaling.

Neurotrophin 3 is the third member of the neurotrophin family. Neurons responsive to NT-3 include cells in both the peripheral and central nervous systems. Among responsive peripheral cells are neurons of the dorsal root ganglia and nodose ganglion and possibly the sympathetic ganglia. In the central nervous system, NT-3-responsive cells include neurons of the trigeminal mesencephalic nucleus, hippocampus, and dopaminergic and GABAergic cells in the ventral mesencephalon.[17,18,26] Motoneurons of the spinal cord also respond.[21] In the peripheral nervous system, NT-3 mRNA is found in muscle. In the central nervous system, high levels of NT-3 mRNA are found in the hippocampus and cerebellum. Neurotrophin 3 mRNA has also been detected in the olfactory bulb, neocortex, diencephalon, midbrain, and spinal cord.[21,24] By in situ hybridization, NT-3 mRNA has been localized to neurons,[24] including embryonic motoneurons.[21,27] Like other neurotrophins, NT-3 binds to $p75^{NGFR}$. Interestingly, it acts by binding to yet another member of the trk family, trkC (Figure 1). Gene expression for trkC is widespread in the central nervous system, with high levels of trkC mRNA in the hippocampus, neocortex, and cerebellum.[28] Messenger RNA of trkC is also found in the ventral mesencephalon[18] and in motoneurons.[21] Like trkB, there are truncated and therefore presumably nonsignaling forms of trkC.[6]

Neurotrophin 4/5 is the most recently discovered member of the neurotrophin gene family. Cells in the peripheral nervous system that are responsive to NT-4/5 include trigeminal, dorsal root ganglion, jugular, sympathetic, and nodose ganglion neurons.[17,29] In the central nervous system, hippocampal neurons and dopaminergic and GABAergic neurons of the ventral mesencephalon respond,[18,26] as do motoneurons.[21] Messenger RNA of NT-4/5 is found in muscle and other peripheral tissues[16] and in the central nervous system. The highest amounts in the central nervous system were found in pons or medulla, hypothalamus, thalamus, and cerebellum.[24] Neurotrophin 4/5 is known to activate the trkB receptor (Figure 1).

The discovery of NGF as a target-derived trophic factor for peripheral neurons has had a major influence on studies directed at understanding its expression and actions in both the peripheral and central nervous systems. In general, data for the expression of NGF and trkA indicate that NGF produced in the target of developing and mature neurons acts through trkA to maintain the survival and enhance the differentiation of responsive neurons. The situation may be more complex for the other neurotrophins. In some instances, a target-derived trophic relationship can be inferred. In others, it appears that autocrine or paracrine relationships may hold. An example of the latter is found in the hippocampus, where it has been shown that certain hippocampal neurons contain mRNA of both BDNF and trkB.[24] A further complication is the production of truncated versions of both trkB and trkC. It will be interesting to further define and characterize the trophic relationships that neurotrophins use to influence the survival and function of their responsive cells.

Ciliary Neurotrophic Factor

During the past few years exciting developments have occurred with respect to another neurotrophic factor called ciliary neurotrophic factor (CNTF). This factor was first purified as a substance extracted from intraocular tissues that supported the survival of ciliary ganglion neurons.[30] In its activity on ciliary neurons and by physicochemical characteristics, the partially purified factor was found to be distinct from NGF. An enriched source of CNTF was found in the adult rat sciatic nerve, and a modified purification protocol was used to purify CNTF activity from this source.[30] This allowed sequencing studies to be done, and eventually CNTF was cloned in several laboratories.[31-33] As predicted from the complementary DNA (cDNA) sequences in rats, rabbits, and humans, CNTF is a protein of 200 amino acids with a molecular weight of 22,700. The availability of purified recombinant protein considerably enhanced studies of the biologic activity of CNTF. Responsive cells of the peripheral nervous system include ciliary ganglion neurons, dorsal root ganglia neurons, sympathetic neurons, and adrenal chromaffin cells. In the central nervous system, hippocampal neurons, forebrain cholinergic neurons, and other populations, including spinal cord motoneurons, are responsive.[17,30] The survival of motoneurons is enhanced by CNTF both in vitro and in vivo.[34-36] In recent studies, disruption of the CNTF gene was shown to produce progressive atrophy and loss of motoneurons.[37] Interestingly, oligodendroglial progenitor cells, referred to as 02A cells, respond to CNTF, as do mature oligodendrocytes.[38]

As indicated, peripheral nerves serve as a rich source for CNTF. The production of CNTF in Schwann cells explains the levels present.[30] An interesting feature of CNTF is the absence of a signal sequence. The meaning of this finding is uncertain, but the suggestion has been made that CNTF release may normally require cell injury with membrane disruption. Ciliary neurotrophic factor-like activity can be shown in stumps affixed to a lesioned nerve in the first few days after a nerve lesion.[30] In the central nervous system astrocytes may produce CNTF,[39] and the levels of production may increase after injury. Thus, it has been suggested that CNTF may play the role of a lesion factor.[30]

The actions of this neurotrophic factor include enhanced neuronal survival. This has been most dramatically demonstrated in studies in vivo. Axotomy of the facial nerve of newborn rats normally results in a substan-

tial loss of motoneurons in the facial nucleus. The application of CNTF to the proximal stump prevented the loss of most neurons.[35] Ciliary neurotrophic factor enhances the cholinergic differentiation of neonatal sympathetic neurons, an effect that parallels the actions of another neurotrophic factor called leukemia inhibitory factor (LIF).[30] These two factors are biochemically distinct, but activate similar receptors (discussed later). An interesting feature of CNTF action in the central nervous system is its ability to prevent the death of axotomized medial septal neurons, both cholinergic and noncholinergic in type. Interestingly, although CNTF promoted the survival of cholinergic neurons that are marked by $p75^{NGFR}$, it did not maintain the activity of choline acetyltransferase, its neurotransmitter synthetic enzyme.[30]

Experiments to identify the CNTF receptor led to the discovery that a specific CNTF binding protein, designated CNTF receptor α (CNTFRα), was homologous to a receptor for the hematopoietic cytokine, interleukin-6 (IL-6).[40] This finding led to several developments. The first was a structural analysis that suggested that IL-6 and CNTF were themselves distantly related and members of a larger family of cytokines that includes LIF, granulocyte colony-stimulating factor, and oncostatin M. All of these factors were known to activate similar intracellular signaling events, and there was the possibility that they used similar receptor systems. Subsequently it was found that CNTF, IL-6, LIF, and oncostatin M do share signal transducing receptor components (Figure 2).[40] All of them use gp130, a transmembrane protein initially identified as the IL-6 signal transducer. In addition, CNTF, LIF, and oncostatin M require a second signal transducer known as LIFRβ. Specificity of CNTF actions arises from its binding to the α-receptor component, together with LIFRβ and gp130. Receptor activation arises from the formation of a heterodimer between LIFRβ and gp130 (Figure 2). The signal is transduced through the activation of a family of cytoplasmic tyrosine kinases known as the Jak-Tyk family. Activation of these kinases leads to the initiation of the signaling cascade in the responsive cell, which includes the phosphorylation of other cellular proteins.[41] One of these is called acute-phase response factor. Acute-phase response factor is a latent cytoplasmic transcription factor that is rapidly activated in response to CNTF.[42]

Distribution of the CNTFRα receptor should define possible sites of CNTF action. In fact, through in situ hybridization histochemistry, CNTFRα has been localized to all the known neuronal targets of CNTF, including neurons in the peripheral ganglia and spinal cord motoneurons.[40] An interesting possibility arises from the structure of CNTFRα. This is a glycosylphosphatidylinositol-linked protein. This linkage is susceptible to enzymatic cleavage, and soluble CNTFRα can be found in cerebrospinal fluid.[40] It is known that cells bearing gp130 and LIFRβ that are normally not responsive to CNTF can be induced to respond by adding CNTF with soluble

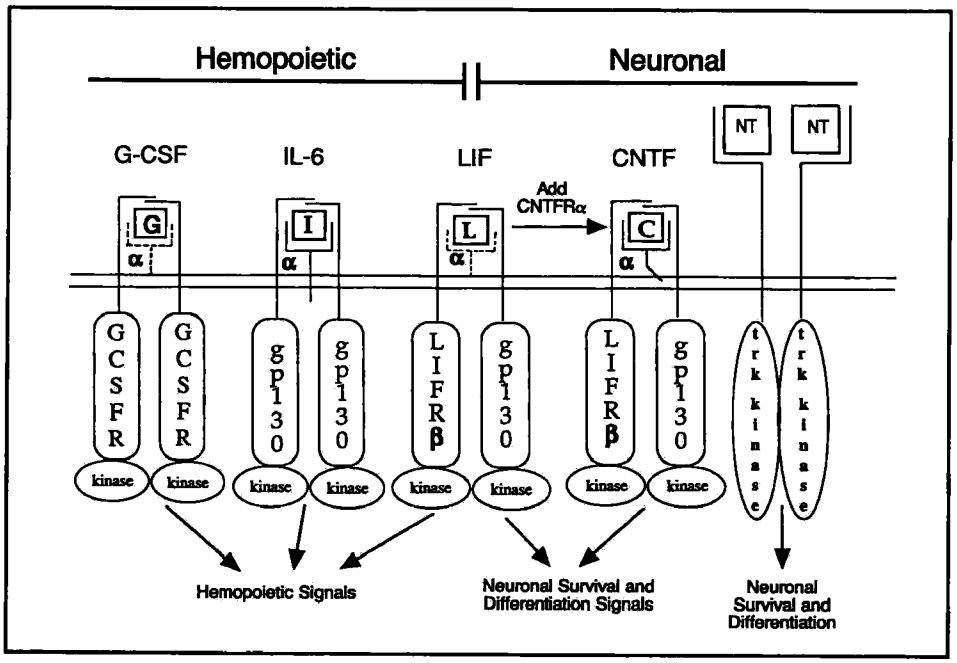

Figure 2.—Models are shown of granulocyte colony-stimulating factor (G-CSF [G]), interleukin-6 (IL-6 [I]), leukemia inhibitory factor (LIF [L]), and ciliary neurotrophic factor (CNTF [C]) receptor complexes (GCSFR, gp130, LIFRβ, and CNTFRα) compared with the trk receptor tyrosine kinases used by neurotrophins (NT) (adapted from Ip and Yancopoulos[40]). The cytokine receptors are depicted as either homodimers or heterodimers of β (gp130 and LIFRβ) components. Complex formation, including the dimerization of β-subunits, only occurs in response to ligand, and results in activation of associated Jak-Tyk tyrosine kinases. In the model, CNTFRα converts a functional LIF receptor complex into a functional CNTF receptor complex. Possible α components for G-CSF and LIF receptor complexes are indicated by dashed lines.

CNTFRα.[43] This suggests two possibilities. First, following the release of CNTFRα from the neuronal membrane, it could act to bind CNTF and prevent it from binding to its receptor on neurons. Alternatively, after binding to CNTF, CNTFRα could carry it to cells expressing gp130 and LIFRβ and induce a response. This analysis suggests a complexity and spectrum of CNTF actions that may be different from those of the neurotrophins.

Other Growth Factors of Interest

The insulin gene family comprises the genes for insulin and the insulinlike growth factors I and II (IGF-I and IGF-II). There is increasing interest in the actions of these molecules in the nervous system. Insulinlike growth factor I is of considerable interest. The mRNA for IGF-I is present in the target zones of trigeminal and sympathetic neurons during the innervation period. It is also found in projection neurons in the maturing sensory and cerebellar relay systems and in nonpyramidal cells of the hippocampus and cerebral cortex. It may also be synthesized by Purkinje cells in the developing cerebellum.[44] There is regulation of IGF-I mRNA levels during development with differences from region to region in the brain. Support for an IGF-I role in developmental regulation of neuronal function and survival comes from in vitro studies showing that IGF-I can induce neurite outgrowth in cultured motor, sensory, and sympathetic neurons. In addition, cortical neurons have been shown to respond, and IGF-I induces oligodendrocyte differentiation and myelin synthesis.[44] Suggesting a role for IGF-I in the development of the neuromuscular junction, IGF-I mRNA levels increase in muscle between embryonic day 14 and birth. Postnatal inhibition of IGF-I mRNA may contribute to the elimination of polyneuronal innervation.[45]

Insulin and the IGFs elicit their actions by binding to specific receptors on the surface of responsive cells. The receptors for insulin and IGF-I are similar.[44] Each is a disulfide-linked heterotetramer consisting of two α- and two β-subunits. The two α-subunits bind the ligand through a cystein-rich domain. These subunits lie entirely outside the cell. The β-subunits, which are linked by disulfide bridges to the α-subunits, contain a short extracellular domain and a transmembrane segment, followed by a cytoplasmic domain. The cytoplasmic domain contains an adenosine triphosphate-binding site and a tyrosine kinase domain. These receptors are formally similar to those defined earlier for the neurotrophins. Activation of the receptor occurs through ligand binding to the α-subunit with a subsequent conformational change in the β-subunits leading to receptor tyrosine kinase activation and autophosphorylation. This is followed by the activation of various intracellular substrates through phosphorylation and the initiation of a cascade of events leading to the biologic response. The IGF-II receptor is distinct from that for insulin and IGF-I. The IGF-II receptor is a single polypeptide chain with a large extracellular domain and a short cytoplasmic domain. Signaling through this receptor may involve glycine protein activation. The concentration of IGF-I receptors peaks in the brain during fetal development. Examining the postnatal and mature brain, IGF-I receptor mRNA is found in sensory and cerebellar relay systems, in the frontal cortex, Ammon's horn, the amygdaloid nuclei, and the suprachiasmatic nucleus. Autoradiography for IGF-I receptors shows rather dense labeling in the olfactory bulb, cerebellum, choroid plexus, and other sites including neocortex.[44] Thus, both IGF-I and its receptor are widely distributed.

Recently a glial cell line-derived neurotrophic factor (GDNF) for midbrain dopaminergic neurons has been described.[46] This potent factor was secreted by a rat glial cell line. It was purified to homogeneity and shown to promote dopamine uptake in cultures of neurons from the midbrain. Both rat and human cDNA for GDNF were cloned using probes based on the amino terminal sequence of purified GDNF. The protein predicted from the sequence data is a secreted molecule of 134 amino acids. It apparently exists as a disulfide-bonded homodimer and is distantly related to members of the transforming growth factor-β superfamily. In midbrain cultures, GDNF promoted the survival and differentiation of dopaminergic neurons. These effects appeared to be relatively specific in that GDNF did not act on GABAergic or on serotonergic neurons.[46]

Animal Models of Disease Treated by Neurotrophic Factors

The remarkable ability of neurotrophic factors to stimulate the survival and differentiation of neurons has suggested their use as trophic agents in diseases. Given advances in understanding the actions of these factors and the distribution of their receptors, we can now begin to develop a list of neurologic disorders and a corresponding list of factors that may prove useful (Table 2). Important diseases that might respond to neurotrophic factors are Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Peripheral neuropathy also suggests itself. Clinical trials are currently underway for amyotrophic lateral sclerosis and vincristine-induced peripheral neuropathy. At the same time, ongoing experiments in animal models of disease encourage the view that neurotrophic factors can be used to treat neurologic disorders.

Nerve Growth Factor Treatment of Taxol Neuropathy

Interesting studies have been done on NGF effects on the toxic neuropathy produced by the chemotherapeutic agent taxol.[47] Taxol has been used against solid tumors such as malignant melanoma and ovarian carcinoma. Unfortunately, its use induces a toxic sensory neuropathy, and this phenomenon has limited its clinical usefulness. The effects of NGF on sensory neurons both in vivo and in vitro encouraged a study in which this trophic factor was used to attempt to prevent taxol neuropathy in vivo.

For these studies an animal model for taxol neuropathy in mice was established. Taxol was administered to the mice intraperitoneally for six days, and they were tested three days thereafter for changes in sensory function. To assess thermal pain threshold, a modification of a

TABLE 2.—*Neurologic Disorders and Candidate Neurotrophic Factors for Therapy*

Disorder	*Neurotrophic Factor**
Peripheral neuropathy	
Sensory neurons	NGF, BDNF, NT-3, NT-4/5
Sympathetic neurons	NGF, FGF-2
Parasympathetic neurons	CNTF
Amyotrophic lateral sclerosis	
Motoneurons	CNTF, BDNF, NT-4/5, IGF-1
Alzheimer's disease	
Basal forebrain cholinergic neurons	NGF
Neocortical neurons	BDNF, NT-3, NT-4/5
Hippocampal neurons	BDNF, NT-3, NT-4/5
Parkinson's disease	
Dopaminergic neurons	GDNF, BDNF, NT-4/5, FGF-1, FGF-2, IGF-I
Huntington's disease	
Striatal interneurons	BDNF, NT-4/5
Striatal cholinergic neurons	NGF

*See Table 1 for abbreviations of neurotrophic factors.

standard tail-flick test was used. Mice were partially restrained, and their tails were placed in a beaker of water. The temperature of the water was increased in increments, and the experimenters observed the temperature at which the mouse flicked its tail out of the water. They were able to show that taxol significantly increased the temperature at which tail-flick occurred. When NGF was administered with taxol, there was no significant difference from control animals in tail-flick temperature. Administering taxol was also found to reduce the mean level of substance P in the dorsal root ganglia. Substance P marks a portion of the NGF responsive neurons in the ganglia. Remarkably, NGF prevented the taxol-mediated decrease in substance P levels. Electrophysiologic studies were also carried out, and compound sensory amplitudes were depressed in the taxol-treated subjects. The coadministration of NGF prevented the decrease. There was little if any effect of taxol on the distal latency recorded in the caudal nerve.[47]

These studies demonstrate clearly that NGF can prevent the effects of taxol on measures of sensory nociceptive function. How NGF exerts its effects is uncertain. One possibility is that it in some way directly counteracts taxol's effects. Another possibility is that NGF protects neurons through some indirect means. An interesting possibility raised by investigators is that taxol's effects on the cytoskeleton may in some way inhibit retrograde transport of the signal normally arising from NGF binding to its receptors in the periphery of sensory neurons.[47] If this were the case, then the administration of exogenous nerve growth factor could correct this deficiency by binding to receptors on or near the cell body. These studies suggest that NGF may be used to limit the dose-related toxicity of taxol and thus to facilitate the use of this drug in cancer patients. Conceivably, NGF or other neurotrophic factors acting on peripheral neurons could be used to augment the clinical usefulness of other cancer chemotherapeutic agents that produce peripheral nerve injury.

Ciliary Neurotrophic Factor and Motoneuron Disease

Several mouse mutants have been used to model motoneuron disease. The pmn/pmn mouse is an autosomal recessive mutant with progressive motor neuronopathy.[48] In homozygotes, paralysis of the hind limbs begins during the third week of life. The forelimbs become weak thereafter, and all mice die between six and seven weeks following birth. Histologic study of muscle shows neurogenic atrophy. Axonal degeneration is seen, which appears to start at motor end plates; it is found predominantly in sciatic nerve and its branches and in the phrenic nerve. Unaffected nerve fibers are normally myelinated, and sensory axons are spared. In the ventral horns, motoneurons show a reduction in cell size, then chromatolysis, and finally cell death. The gene responsible for this disorder is unknown.

On the basis of CNTF effects on motoneurons in vivo and in vitro, including the ability of this factor to prevent lesion-mediated degeneration of rat motoneurons during early postnatal life,[35] investigators pursued the hypothesis that CNTF could be used to prevent motoneuron degeneration in pmn/pmn mice.[49] It was first demonstrated that abnormalities in CNTF production were unlikely to cause the disease. Messenger RNA levels of CNTF in the sciatic nerve of these subjects were similar to those in normal mice. Also, extracts of nerves showed an equivalent amount of CNTF biologic activity. To administer CNTF to these subjects, a stable cell line was created by a transfection of mouse D3 cells with a construct in which CNTF genomic DNA was cloned 3′ to a cDNA fragment coding for the first 20 amino acids of the mouse prepro/NGF sequence, which includes the entire signal peptide for this molecule. The thought was that this construct would allow both synthesis and secretion of CNTF in these animals. Experiments in which CNTF expression was tested in vitro showed that CNTF activity was readily released into the culture medium. After intraperitoneal

administration of these cells on postnatal day 21, intraabdominal tumors were produced, and it was shown that CNTF was released into the serum of these animals. The production of CNTF in vivo was associated with enhanced survival and with enhanced motor performance compared with untreated or mock-treated pmn/pmn mice. Both treated and untreated mice were killed between postnatal days 40 and 48. Examination of the facial nucleus showed that the number of motoneurons was greatly decreased in pmn/pmn mice and that treatment with CNTF had a large protective effect. Thus, whereas in untreated mice only 60% of neurons remained, in treated mice, 86% of cells were present. Also, the number of axons in the phrenic nerve was substantially increased by CNTF treatment.[49]

The data show that CNTF rescues motoneurons from degeneration in pmn/pmn mice. They suggest that CNTF might be used to prevent motoneuron death and dysfunction in motoneuron disease in humans. The possibility is raised that other factors active on motoneurons might also be useful, either given separately or in conjunction with CNTF.

Nerve Growth Factor and the Trisomy 16 Mouse

The focus of work in our laboratory has been on understanding the natural processes of normal and diseased basal forebrain cholinergic neurons. Special emphasis has been placed on the response of these neurons to neurotrophic factors, especially NGF. Basal forebrain cholinergic neurons atrophy and die in patients with Alzheimer's disease, and it is known that these neurons are likely to contribute substantially to learning and memory deficits in these patients.[50] In developing an animal model for Alzheimer's disease, we made note of the interesting observation that the neurologic abnormalities of Alzheimer's disease universally develop at an early age in patients with the Down syndrome (trisomy 21). We reasoned that an animal model for Down syndrome might recapitulate important features of the neuropathogenesis of Alzheimer's disease and allow us to pursue the mechanism for the degeneration of basal forebrain cholinergic neurons. The trisomy 16 mouse is a genetic model for human Down syndrome. Mouse chromosome 16 contains a cluster of genes and loci that are also located on the proximal arm of human chromosome 21. These include the amyloid precursor protein, one of the glutamate receptor genes, and superoxide dismutase 1. Fetal trisomy 16 mice demonstrate phenotypic features seen in Down syndrome, including endocardial cushion defects and hematologic and immunologic abnormalities.

We hypothesized that cholinergic neurons of trisomy 16 murine basal forebrain would show degenerative changes over time in vivo. To compare cholinergic neurons from trisomy 16 and from normal mice, we transplanted fetal basal forebrain from trisomy 16 mice and from control fetuses into the hippocampus of normal mice. The hippocampus is the normal target of these neurons. Transplantation experiments showed that both trisomy 16 and control grafts survived for long periods in vivo. Interestingly, while cholinergic neurons appeared normal in both trisomy 16 and control transplants after one month, after six months there was clear-cut atrophy of trisomy 16 neurons. Atrophy was specific for cholinergic neurons.[51] To determine whether NGF could reverse this atrophy, NGF was administered by intraventricular cannulae between 5½ and 6 months of age. Vehicle injection served as the control. Animals were killed at 6 months of age, and grafts were assessed by immunostaining for choline acetyltransferase, the neurotransmitter synthetic enzyme for cholinergic neurons. As in earlier studies, there was atrophy of trisomy 16 cholinergic neurons relative to control neurons. The mean cross-sectional area was reduced by about 20% in vehicle-treated subjects. Following NGF infusion, trisomy 16 cholinergic neurons were significantly enlarged. Remarkably, both trisomy 16 and control neurons treated with NGF were substantially larger than control cholinergic neurons in vehicle-treated animals. The increase for trisomy 16 cells was to a size 29% greater than vehicle-treated control cholinergic neurons. There was no significant difference between the size of NGF-treated trisomy 16 and control neurons.[52] In follow-up studies we asked whether NGF levels were different in the hippocampus of animals that received trisomy 16 cells versus those that received control cells. No difference in NGF levels was apparent. Indeed, the size of the host basal forebrain cholinergic neurons, which have their axons in the same hippocampal territories as transplanted cells, was normal on both the side of the trisomy 16 transplant and the side of the control transplant. This suggests that the level of NGF available to trisomy 16 and control neurons was equivalent. We next asked whether NGF receptors continued to be expressed in neurons of trisomy 16 mice. We found evidence of both trkA and $p75^{NGFR}$ expression in both control and trisomy 16 neurons.[52] The levels of receptor expression and binding are now being addressed.

Using the trisomy 16 mouse model of the Down syndrome, we have shown that NGF can reverse genetically determined neuronal atrophy. Further analysis of this model may give insight into pathways that lead to neuronal degeneration in vivo and establish how NGF and other neurotrophic factors could be used to prevent dysfunction and death of these neurons in Alzheimer's disease.

Pursuing the Mechanisms of Neurodegeneration

The studies cited in this article give evidence that neurotrophic factors can prevent or reverse the degeneration and dysfunction of neurons caused by environmental and genetic insults. In addition to suggesting novel forms of therapy for these disorders, they raise the question of whether neurotrophic factors may be implicated in the pathogenesis of nervous system disorders. One possibility is that a primary deficiency of the neurotrophic factor is at the root of the disease. The examples cited earlier provide evidence that this is not required to produce such disorders. Indeed, in the case of the trisomy 16 mouse

transplants, atrophy occurred over a several-month period in vivo in the presence of what appear to have been normal levels of NGF. Thus, it would appear that an intrinsic abnormality in trisomy 16 cells was responsible for the changes that were demonstrated. No abnormality of a trophic factor has yet been clearly implicated in a disease state. Nevertheless, recent exciting work on mice in which the genes for either NGF or BDNF have been knocked out show unequivocally that eliminating the production of individual neurotrophic factors can produce considerable neuronal loss.[53,54]

A second possible explanation for neurodegeneration is that there is some fundamental abnormality in the receptor for a specific neurotrophic factor. Again, there are no disease examples to prove the case, but the possibility can be easily envisioned. A third possibility is that events downstream from the receptor that are important for signaling that leads to a maintenance of neuronal survival and function are altered in diseased neurons. In fact, these two possibilities can be combined in the case of the trisomy 16 mouse to suggest a means by which these neurons degenerate. It is known that NGF must bind to these receptors to activate its responsive cells. It is known also that one response to NGF is for gene expression for its receptors to be induced in these neurons.[55] Thus, even a subtle decrease in the ability of NGF receptors to be bound or activated by NGF, or in any of the many downstream events leading to the regulation of receptor gene expression, could in time result in fewer receptors at the cell surface. This situation could create progressive neuronal atrophy and conceivably even death of these cells in the presence of normal levels of NGF.

Finally, it is worth mentioning that the response of neurons to neurotrophic factors could be substantially different in patients with disease. The possibility can be envisioned that both beneficial and deleterious effects could arise from neurotrophic factor treatment of diseased neurons. Although it will be difficult to predict in advance in which diseases this may occur, these observations argue strongly for carrying out studies in realistic animal models of disease before human trials are conducted. In the case of the trisomy 16 mouse, NGF treatment did not appear to injure the responding neurons and created no new disorder.[52] Thus, for NGF there is no evidence that a deleterious effect would accompany its actions on diseased basal forebrain cholinergic neurons.

The observations cited in this article make it clear that there is a great deal of work yet to do to understand and to exploit the therapeutic potential of neurotrophic factors. Studies on the biology of these factors must proceed concurrently with studies designed to understand the mechanism of their action in reversing neurodegeneration.

Clinical Trials Using Neurotrophic Factors

There are several ongoing trials in which neurotrophic factors are being used to attempt to inhibit the dysfunction and death of neurons in specific neurologic disorders. Genentech has sponsored studies examining NGF for the treatment of peripheral neuropathy. A Phase I trial is now complete, and there are plans for testing NGF effects in diabetic neuropathy later this year. Genentech has also announced an interest in sponsoring trials of NGF in Alzheimer's disease. The neurotrophic factor would be delivered by intraventricular cannula. This ambitious trial is scheduled to begin within the next year. Amgen-Regeneron Partners are sponsoring a project to examine the effects of BDNF in amyotrophic lateral sclerosis. Other trials of neurotrophic factors have also been directed at this neurologic disorder. A Regeneron-sponsored Phase III trial using CNTF at either a high or a low dose was recently completed. The final analysis of the data is pending, but based on an interim analysis, it is not expected that CNTF-treated patients will be improved relative to placebo-treated controls. This is thought to be due, at least in part, to impaired neuromuscular function resulting from untoward side effects, which included anorexia, weight loss, nausea, and cough. A second trial for CNTF in amyotrophic lateral sclerosis is being sponsored by Synergen. In this trial, CNTF is being used at a lower dose. There are no results as yet available. Cephalon is also sponsoring trials in this disorder, using IGF-I in a Phase II/III North American trial that should be completed by the end of 1994. Preliminary data analysis is expected to be available in early 1995. Cephalon is sponsoring a similar trial in Europe; preliminary data should be available by mid- to late-1995. Finally, Cephalon is planning a Phase II trial using IGF-I in vincristine neuropathy.

REFERENCES

1. Levi-Montalcini R, Hamburger V: Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J Exp Zool 1951; 116:321-362

2. Levi-Montalcini R, Hamburger V: A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. J Exp Zool 1953; 123:233-288

3. Longo FM, Holtzman DM, Grimes ML, Mobley WC: Nerve growth factor: Actions in the peripheral and central nervous systems, *In* Loughlin S, Fallon J (Eds): Neurotrophic Factors. San Diego, Calif, Academic Press, 1993, pp 209-256

4. Chao MV: Neurotrophin receptors: A window into neuronal differentiation. Neuron 1992; 9:583-593

5. Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF: The trk proto-oncogene product: A signal transducing receptor for nerve growth factor. Science 1991; 252:554-558

6. Grimes M, Zhou J, Li Y, Holtzman D, Mobley WC: Neurotrophin signalling in the nervous system. Semin Neurosci 1993; 5:239-247

7. Saltiel AR, Ohmichi M: Pleiotropic signaling from receptor tyrosine kinases. Curr Opin Neurobiol 1993; 3:352-359

8. Barde YA, Edgar D, Thoenen H: Purification of a new neurotrophic factor from mammalian brain. EMBO J 1982; 1:549-553

9. Ernfors P, Ibanez CF, Ebendal T, Olson L, Persson H: Molecular cloning and neurotrophic activities of a protein with structural similarities to beta-NGF: Developmental and topographical expression in the brain. Proc Natl Acad Sci USA 1990: 87:5454-5458

10. Hohn A, Leibrock J, Bailey K, Barde YA: Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 1990; 344:339-341

11. Maisonpierre PC, Belluscio L, Squinto S, et al: Neurotrophin-3: A neurotrophic factor related to NGF and BDNF. Science 1990; 247:1446-1451

12. Rosenthal A, Goeddel DV, Nguyen T, et al: Primary structure and biological activity of a novel human neurotrophic factor. Neuron 1990; 4:767-773

13. Jones KR, Reichardt LF: Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc Natl Acad Sci USA 1990; 87:8060-8064

14. Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A: Neurotrophin-5: A novel neurotrophic factor that activates trk and trk B. Neuron 1991; 7:857-866

15. Hallböök F, Ibanez C, Persson H: Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 1991; 6:845-858

16. Ip NY, Ibanez CF, Nye SH, et al: Mammalian neurotrophin 4: Structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci USA 1992; 89:3060-3064

17. Korsching S: The neurotrophic factor concept: A reexamination. J Neurosci 1993; 13:2739-2748

18. Hyman C, Juhasz M, Jackson C, et al: Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. J Neurosci 1994; 14:335-347

19. Yan Q, Elliott J, Snider WD: Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. Nature 1992; 360:753-755

20. Sendtner M, Holtmann B, Kolbeck R, Thoenen H, Barde YA: Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. Nature 1992; 360:757-759

21. Henderson CE, Camu W, Mettling C, et al: Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature 1993; 363:266-270

22. Koliatsos VE, Clatterbuck RE, Winslow JW, Cayouette MH, Price DL: Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. Neuron 1993; 10:359-367

23. Lindsay RM: Brain-derived neurotrophic factor: An NGF-related neurotrophin, *In* Loughlin S, Fallon J (Eds): Neurotrophic Factors. San Diego, Calif, Academic Press, 1993, pp 257-284

24. Persson H: Neurotrophin production in the brain. Semin Neurosci 1993; 5:227-237

25. Ringstedt T, Lagercrantz H, Persson H: Expression of members of the trk family in the developing postnatal rat brain. Dev Brain Res 1993; 72:119-131

26. Ip NY, Li Y, Yancopoulos GD, Lindsay RM: Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J Neurosci 1993; 13:3394-3405

27. Schecterson LC, Bothwell M: Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron 1992; 9:449-463

28. Lamballe F, Klein R, Barbacid M: trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 1991; 66:967-979

29. Davies AM, Horton A, Burton LE, Schmelzer C, Vandlen R, Rosenthal A: Neurotrophin-4/5 is a mammalian-specific survival factor for distinct populations of sensory neurons. J Neurosci 1993; 13:4961-4967

30. Manthorpe M, Louis JC, Hagg T, Varon S: Ciliary neurotrophic factor, *In* Loughlin S, Fallon J (Eds): Neurotrophic Factors. San Diego, Calif, Academic Press, 1993, pp 443-473

31. Lin LF, Mismer D, Lile J, et al: Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science 1989; 246:1023-1025

32. Stockli KA, Lottspeich F, Sendtner M, et al: Molecular cloning, expression and regional rat ciliary neurotrophic factor. Nature (London) 1989; 342:920-923

33. Lam A, Fuller F, Miller J, et al: Sequence and structural organization of the human gene encoding ciliary neurotrophic factor. Gene 1991; 102:271-276

34. Arakawa Y, Sendtner M, Thoenen H: Survival effect of ciliary neurotrophic factor (CTNF) on chick embryonic motoneurons in culture: Comparison with other neurotrophic factors and cytokines. J Neurosci 1990; 10:3507-3515

35. Sendtner M, Kreutzberg GW, Thoenen H: Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 1990; 345:440-441

36. Oppenheim RW, Prevette D, Qin-Wei Y, Collins F, MacDonald J: Control of embryonic motoneuron survival in vivo by ciliary neurotrophic factor. Science 1991; 251:1616-1618

37. Masu Y, Wolf E, Holtmann B, Sendtner M, Brem G, Thoenen H: Disruption of the CNTF gene results in motor neuron degeneration. Nature 1993; 365:27-32

38. Louis JC, Magal E, Takayama S, Varon S: CNTF protection of oligodendrocytes against natural and tumor necrosis factor-induced death. Science 1993; 259:689-692

39. Stockli KA, Lillien LE, Naher-Noe M, et al: Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. J Cell Biol 1991; 115:447-459

40. Ip NY, Yancopoulos GD: Receptors and signaling pathways of ciliary neurotrophic factor and the neurotrophins. Semin Neurosci 1993; 5:249-257

41. Stahl N, Boulton TG, Farruggella T, et al: Association and activation of Jak-Tyk kinases by CNTF-LIF-IL-6 β-receptor components. Science 1994; 263:92-95

42. Lutticken C, Wegenka UM, Yuan J, et al: Association of transcription factor APRF and protein kinase Jak 1 with the interleukin-6 signal transducer gp130. Science 1994; 263:89-92

43. Davis S, Aldrich TH, Ip NY, et al: Released form of CNTF receptor α component as a soluble mediator of CNTF responses. Science 1993; 259:1736-1739

44. LeRoith D, Roberts CTJ, Werner H, et al: Insulin-like growth factors in the brain, *In* Loughlin S, Fallon J (Eds): Neurotrophic Factors. San Diego, Calif, Academic Press, 1993, pp 391-414

45. Ishii DN: Neurobiology of insulin and insulin-like growth factors, *In* Loughlin S, Fallon J (Eds): Neurotrophic Factors. San Diego, Calif, Academic Press, 1993, pp 415-442

46. Lin LFH, Doherty DH, Lile JD, Bektesh S, Collins F: GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 1993; 260:1130-1132

47. Apfel SC, Lipton RB, Arezzo JC, Kessler JA: Nerve growth factor prevents toxic neuropathy in mice. Ann Neurol 1991; 29:87-90

48. Schmalbruch H, Jensen HJS, Bjaerg M, Kamieniecka Z, Kurland L: A new mouse mutant with progressive motor neuronopathy. J Neuropathol Exp Neurol 1991; 50:192-204

49. Sendtner M, Schmalbruch H, Stockli KA, et al: Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. Nature 1992; 358:502-504

50. Coyle JT, Price DL, DeLong MR: Alzheimer's disease: A disorder of cortical cholinergic innervation. Science 1983; 219:1184-1190

51. Holtzman DM, Li Y, DeArmond SJ, et al: A mouse model of neurodegeneration: Atrophy of basal forebrain neurons in Ts 16 transplants. Proc Natl Acad Sci USA 1992; 89:1383-1387

52. Holtzman DM, Li Y, Chen K, et al: Nerve growth factor reverses neuronal atrophy in a Down syndrome model of age-related neurodegeneration. Neurology 1993; 43:2668-2673

53. Crowley C, Spencer SD, Nishimura MC, et al: Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 1994; 76:1001-1011

54. Jones KR, Fariñas I, Backus C, Reichardt LF: Targeted disruption of the BDNF gene perturbs brain and sensory neuron development. Cell 1994; 76:989-999

55. Holtzman DM, Li Y, Parada LF, et al: p140^{trk} mRNA marks NGF-responsive forebrain cholinergic neurons: Evidence that trk gene expression is induced by NGF. Neuron 1992; 9:465-478